SEX HORMONES REGULATE HERG K^+ CHANNEL TRAFFICKING AND FUNCTION

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LIST OF PUBLICATION

1. **Wu ZY**, Chen K, Haendler B, McDonald TV, Bian JS. Stimulation of N-terminal truncated isoform of androgen receptor stabilizes human ether-á-go-go-related gene-encoded potassium channel protein via activation of extracellular signal regulated kinase 1/2. Endocrinology. 2008 Oct;149(10):5061-9. Epub 2008 Jul 3.

2. **Wu ZY,** Yu DJ, Soong TW, Dawe GS, Bian JS. Progesterone impairs HERG trafficking by disruption of intracellular cholesterol homeostasis. J Biol Chem. 2011 Apr 27. [Epub ahead of print]

3. Xu ZS, Wang XY, Xiao DM, Hu LF, Lu M, **Wu ZY**, Bian JS. Hydrogen sulfide protects MC3T3-E1 osteoblastic cells against H_2O_2-induced oxidative damage-implications for the treatment of osteoporosis. Free Radic Biol Med. 2011 May 15;50(10):1314-23. Epub 2011 Feb 24.

4. Chen J, Chen K, Sroubek J, **Wu ZY**, Thomas D, Bian JS, McDonald TV. Post-transcriptional control of HERG potassium channel protein by α-adrenergic receptor stimulation. Mol Pharmacol. 2010 Aug;78(2):186-97. Epub 2010 May 12.

5. Hu LF, Lu M, **Wu ZY**, Wong PT, Bian JS. Hydrogen sulfide inhibits rotenone-induced apoptosis via preservation of mitochondrial function. Mol Pharmacol. 2009 Jan;75(1):27-34. Epub 2008 Oct 2.

ABBREVIATIONS

5α-DHT	5α-dihydrotestosterone
5β-DHT	5β-dihydrotestosterone
ALLN	N-acetyl-L-leucyl-L-leucyl-L-norleucinal
AR	androgen receptor
AR45	Cardiac splice isoform of androgen receptor
cAMP	cyclic adenosine monophosphate
CHO cell	Chinese hamster ovary cells
CHX	cycloheximide
cNBD	cyclic nucleotide binding domain
CNS	central nervous system
EC50	half maximal effective concentration
ER	endoplasmic reticulum
ERG	ether-a-go-go related gene
ERα	estrogen receptor α
HEK293 cell	human embryo kidney cell
HERG	human ether-a-go-go related gene
Hsc70	Heat shock cognate protein 70
Hsp70	heat shock protein 70
Hsp90	heat shock protein 90
Ikr	rapidly activating delayed rectifier K+ current
Iks	slow activating delayed rectifier K+ current
I_{Kv}	voltage-gated K+ current
$I_{Ca,L}$	L-type calcium current
LQTS	The long QT syndrome

miRNA	MicroRNA
MiRP1	MinK-related peptide 1
nAChR	nicotinic acetycholine receptor
P4	Progesterone
PAS domain	Per-ARNT-Sim domain
PIP2	Phosphatidyl inositol bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
QTc interval	the heart rate corrected QT interval
SERCA	sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase
$t_{1/2}$	the half-life time
TdP	torsade de pointes
UPR	unfolded protein response
$V_{1/2}$	The half activation potential
WT	Wild type

SUMMARY

The long QT syndrome (LQTS), which is characterized as prolongation of QT intervals, is an inherited and acquired channelopathy associated with sudden cardiac death due to ventricular arrhythmias. Malfunction of human ether-á-go-go-related gene (HERG) encoded K^+ channel is one of the major causes of LQTS. In the first chapter of this thesis, the physiology of HERG channel including the structure, unique gating and biosynthesis, was reviewed. Then, recent progress on regulatory effects of sex hormones on HERG current was summarized.

Proarrhythmic drugs induce *torsade de pointes* more frequently in women than men. To reveal the mechanism for the gender differences in QT interval and acquired LQTS, I first investigated the effect of androgen and estrogen on HERG proteins. It was found that treatment with estrogen failed to affect HERG channel expression. In contrast, androgens increased HERG protein abundance in the presence of cardiac androgen receptor variant (AR45), but not full length androgen receptor. Confocal microscopy showed that the upregulated HERG proteins were seen in the ER, Golgi complex and plasma membrane without clear preferential colocalization. Chronic androgen treatment also increased HERG K^+ current density in the presence of AR45. Moreover, 5α-DHT increased ERG protein abundance in isolated rabbit cardiac myocytes. The upregulation of HERG protein was due to inhibition of channel degradation, instead of enhancing channel synthesis. In addition, 5α-DHT/AR45 signaling induced phosphorylation of extracellular signaling regulated kinase (ERK1/2). Blockade of ERK1/2 prevented the effect of androgen on HERG protein abundance. In conclusion, these data provide evidence that stimulation of AR45 receptors by androgens upregulates HERG K^+ channel abundance and activity mainly through stabilizing

HERG protein in an ERK1/2 dependent mechanism and suggest a mechanism to explain the sex difference in the long QT syndrome.

Progesterone level increases steadily during pregnancy, and reaches μmol/L level before the delivery. The second part of my work was to investigate the effect of progesterone on HERG channel function. It was found that chronic progesterone treatment decreased the abundance of mature HERG channel in rat neonatal cardiac myocytes and HERG-HEK293 cells. Progesterone also concentration-dependently decreased HERG current density. Immunofluoresence microscopy shows that progesterone preferentially decreased HERG channel protein abundance in the plasma membrane, induced protein accumulation in the dilated ER. Application of sterol binding agent or over-expression of Rab9 rescued the progesterone-induced HERG trafficking defect. Disturbance of intracellular cholesterol homeostasis mimicked the effect of progesterone on HERG channel trafficking. Progesterone may impair HERG channel folding in the ER and/or block its trafficking to the Golgi complex by disrupting intracellular cholesterol homeostasis. This finding may reveal a novel molecular mechanism to explain the QT prolongation during late pregnancy.

In conclusion, this thesis examined how sex hormones regulate HERG K^+ channels with a combination of pharmacological manipulations, confocal microscopy, molecular biology and electrical physiology. This study not only provides a molecular mechanism for the sex differences in QT intervals and drug-induced arrhythmias but also explains the longer QT interval during late pregnancy and the high risk of arrhythmias development in the fetus.

Chapter 1 INTRODUCTION AND LITERATURE REVIEW

Sudden cardiac death causes approximately half of all cardiovascular related deaths, which account for more than 300,000 deaths every year in the United States alone[1-2]. Sudden cardiac death is an unexpected death which happens within one hour from the onset of the cardiac event. Although some cases are results of previous cardiac pathology, many victims are otherwise healthy with no heart disease history and free from heart structure damage. This indicates that the abnormities of cardiac electrical transmission, such as prolonged QT intervals and slower heart rates, may be the cause of some sudden cardiac deaths.

The long QT syndrome (LQTS), which is characterized as prolongation of QT intervals, is an inherited and acquired channelopathy associated with sudden cardiac death due to ventricular arrhythmias. These arrhythmias cause loss of cardiac pump function, and then cut off blood supply to the brain, thus inducing brain death. For this reason, LQTS is potentially fatal and high mortality is noticed. The initial recorded mortality rate is extremely high due to the poor diagnosis in the early years, as only the most severely affected patients can be identified. More recently, the mortality of untreated symptomatic patients was first reported to be 71 %, but later dropped to 20 % for patients treated with beta blocker. The inherited LQTS can be divided into several subgroups based on the type of affected molecules which are listed in the table (1-1).

Overall, the first three kinds of LQTS (LQT1, 2 and 3) are common, and present in more than 90% of inherited LQTS patients. Acquired LQTS is another form of LQTS, which is more likely related to the blockage of rapidly activating delayed rectifier K^+ current (Ikr), whose alpha-subunit is encoded by human ether-a-go-go related gene (HERG).

Table 1-1 The Genes associated with inherited Long QT Syndromes

Type	Gene	Chromosomal localization	Protein	Function	Lose/Gain of function	Prevalence in LQTS patients (%)
LQT1	KCNQ1	11p15.5	Kv7.1/KvLQT1	α-subunit I_{Ks}	Lose	40–55
LQT2	KCNH2	7q35–7q36	Kv11.1/HERG	α-subunit I_{Kr}	Lose	35–45
LQT3	SCN5A	3p21	Nav1.5	α-subunit I_{Na}	Gain	2–8
LQT4	ANK2	4q25–4q27	Ankyrin B	Adaptor (I_{Na-K}, I_{Na-Ca}, I_{Na})	Lose	<1
LQT5	KCNE1	21p22	minK	β-subunit I_{Ks}	Lose	<1
LQT6	KCNE2	21p22	MiRP1	β-subunit I_{Kr}	Lose	<1
LQT7	KCNJ2	17q23.1–17q24	Kir2.1	α-subunit I_{K1}	Lose	<1
LQT8	CACNA1C	12p13.3	Cav1.2	α-subunit I_{Ca}	Gain	<1
LQT9	CAV3	3p25	M-Caveolin	Adaptor (I_{Na})	Lose	<1
LQT10	SCN4B	11q23	Navb4	β-subunit I_{Na}	Lose	<0.1
LQT11	AKAP9	7q21–7q22	Yotiao	Adaptor(I_{Ks})	Lose	<0.1
LQT12	SNTA1	20q11.2	a1-Syntrophin	Scaffolding protein (I_{Na})	Lose	<0.1

The table is reproduced and adopted from Hedley et al., 2009[3].

HERG encoded potassium channel has been extensively studied, not only because it is the first reported gene for cardiac disease, but also because it affects a large number of LQTS patients (around 40% of inherited LQTS patients and a majority of acquired LQTS patients). Next we will discuss the structure, function, and biosynthesis of HERG.

1.1 HERG gene and protein structure

The HERG gene was initially isolated by screening a human hippocampal cDNA library with probes based on mouse eag[4]. HERG gene is located on the chromosome 7 (7q35-36)[5], and the mRNA is 3900 bp long which contains 16 exons and polyA signal (NCBI Reference Sequence: NM_000238.2). According to the website http://www.fsm.it/cardmoc/, 291 HERG mutations have been reported by now. The mutation types include missense, nonsense, deletion, insertion, splice error, frameshift, deletion/frameshift, duplication/inframe, duplication/frameshift, insertion/frameshift. These mutations can cause loss-of-function via affecting HERG channel transcription, translation, trafficking, or channel gating.

HERG encodes a polypeptide with a predicted molecular weight of 127 kDa, which appeared on western blot as bands of 135 kDa and 155 kDa because of the posttranslational modifications (such as glycosylation). The membrane topology of HERG is predicted based on the knowledge of HERG sequence and other ion channel structures (Figure 1-1). HERG potassium channels are transmembrane proteins which contain intracellular N-terminal domain, six transmembrane domains (denoted S1-S6), pore loop, S5-P linker (between domain S5 and pore loop) and C-terminal domain. N-terminal domain is crucial for channel deactivation. S4 domain contains multiple positive charges and is the major part of the voltage sensor. The pore loop contains potassium ion selectivity filter (GYG) and glycosylation site (N598). C-terminal domain contains several phosphorylation sites and trafficking signals.

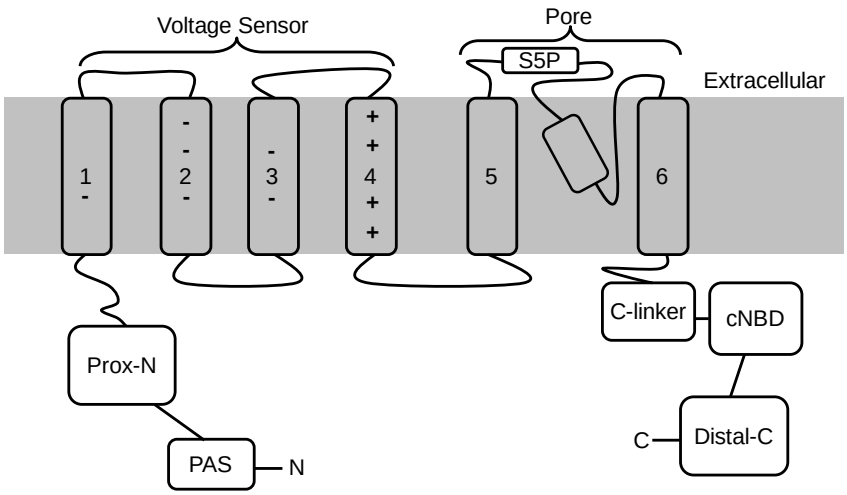

Figure 1-1 Topology of HERG protein

1.2 HERG channel gating

Voltage gated potassium (Kv) channels generally have at least three conformation states: closed, open, and inactivated. Among these states, only the open state is conducting state.

Transition from the closed states to the open states is called activation, and the reverse transition called deactivation. Both processes rely on the conformation changes of transmembrane domain S6. Inactivation of HERG channel relies on the conformation changes around the selectivity filter[6]. The general gating sequence of a Kv channel is a fast activation, slow and voltage-independent inactivation, and slow deactivation. Despite the high homology in amino acid sequence to other voltage-gated potassium channels, HERG channel has unique current kinetics: slower onset of the activation and fast and voltage-dependent of the inactivation. This unique gating is crucial as it can suppress the propagation of the premature beats and prevent arrhythmias triggered by premature beats (Lu et al., 2001).

The mutagenesis studies on HERG channels, either naturally occurring or artificially synthesized mutants shed the light on the relationship between HERG structure and HERG gating [7-11].

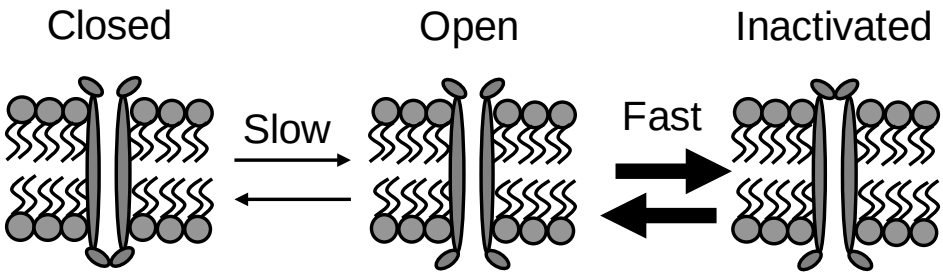

Figure 1-1 HERG gating

1.2.1 Activation

The activation gate of potassium channel is formed by the C-terminal part of the S6 transmembrane helices. These helices form a bundle crossing and prevent potassium ions going through[12]. The gating hinge in HERG channel is formed by ^{655}PVG^{657} instead of the conserved PVP motif in Kv1-Kv4 channels [8, 13]. This is supported by the experiment that

replacement of glycine with proline at the position 657 results in a permanently open channel[8].

For voltage-gated ion channels, S1-S4 voltage sensor can modulate the transition between the closed state and the open state. In HERG channel, the six basic amino acids located in the S4 domain provide positive charges needed for the detection of transmembrane voltage changes. On the other hand, the salt bridges formed between these basic amino acids and acidic amino acids in S1-S3 domains can stabilize the voltage sensor in either open or closed state [14].

Mutations occurred in or close to the voltage sensor may affect its conformation and change HERG channel activation[9, 11].

The molecular basis for the slow onset of activation for HERG channel is not well understood. Studies on the gating currents and fluorescence labeling indicate that the slow voltage sensor movement may contribute to the slow activation of HERG channels[15-16].

1.2.2 Deactivation

The deactivation of HERG channel is mainly regulated by its N-terminal region. Mutations (missense or deletion) in the N-terminal of HERG channel can accelerate channel deactivation, and applying of recombinant N-terminal domain can restore the deactivation[7, 17].

1.2.3 Inactivation

There are two main theories explain the inactivation of voltage-gated potassium channels: N-type inactivation (ball and chain model) and C-type inactivation (collapse of the pore model). The C-type inactivation in HERG channel is unique because of its voltage dependency and fast kinetics.

Several studies on the voltage dependency of HERG inactivation indicate that this voltage sensitivity is not from the voltage sensor of activation. Firstly, mutations in the S5-P linker and pore loop affect inactivation but not activation [18-21]. These data indicate that S5-P linker and pore loop are important for the voltage-dependency of HERG inactivation. Secondly, changes of temperature have opposite effects on activation and inactivation of HERG channel[22].

1.2.4 Conductance/ionic selectivity

The ionic selectivity and channel conductance of HERG channel are mainly determined by the pore loop, especially the selectivity filter. Mutations in this area can change ionic selectivity and channel conductance, if the mutant channels can be inserted into the plasma membrane properly [23-26]. However, the mutations in this area affect HERG channel proper folding and most likely induce trafficking defect [27].

1.3 Modulation of HERG channel gating

The gating of HERG channel can be regulated by the surrounding environment (ions, proteins, and lipids), applied chemicals (blockers and activators), temperature, and modification on the channel (as by protein kinases).

1.3.1 Extracellular ions

K^+: Extracellular potassium ions are important for HERG channel function. Potassium ion free solution is an extreme situation, on which HERG channels will go into an unusual non-conductance state within minutes[28]. This process is reversible and linked with the pore region of HERG channel, but different from that of inactivation or channel blocker E-4031 binding sites[28]. On the other hand, elevation of extracellular potassium ion concentration has no significant effect on the gating of HERG channel[29].

Ca^{2+}/Mg^{2+}: Increasing extracellular Ca^{2+} causes a reduction of HERG currents via depolarizing shifting of voltage dependency of activation, slowing of activation, and accelerating of deactivation [30-31]. The extracellular Mg^{2+} has similar effect as Ca^{2+} [30, 32].

pH: Extracellular pH may affect the charges of amino acids in HERG channel, which in turn affect HERG gating. Extracellular acidification suppresses HERG current via depolarizing shifting of the voltage-dependence of the activation and acceleration of deactivation[29, 33-35].

1.3.2 Blockers and activators

Blockers: Drug blockage on HERG channel is a main cause for drug-induced LQTS, and several drugs as astemizole, probucol and terfenadine have been removed from market because of their high potency of blocking HERG channel. A detailed list of drugs related with LQTS is compiled in a website: http://www.azcert.org/medical-pros/drug-lists/drug-lists.cfm.

Great efforts have been made to study the molecular basis of drug blockage on HERG channel, and the knowledge gained has been applied to the safety of new drug development. It is well accepted that aromatic amino acids tyrosine652 and phenylalanine656 which face the inner cavity, are high affinity binding sites for most drugs[36]. However, the bindings between the channel and drugs are far more complex as the precise binding arrangements require residues within and between different subunits, and the arrangements are likely to change during the process of gating. It is suggested that the inactivated state is the most accessible state for high-affinity binding drugs (reviewed in Perrin et al., 2008[37]).

Activators: A group of HERG channel activators that can potentiate HERG current have been discovered recently. These activators can increase HERG currents via reduction of

HERG inactivation (ICA-105574[38]), slowing of HERG deactivation (RPR260243[39]), facilitation of activation, or the combinations of these effects (PD-307243[40]; A-935142[41]). The molecular basis for the HERG activator is not clear, although S5-P linker and pore loop are reported to be the binding site for HERG channel activator[40].

1.3.3 Lipids

In the plasma membrane, HERG channels are concentrated in the cholesterol and sphingolipid enriched domains[42]. As floating in the membrane lipid "ocean", the function of HERG channel can be modulated by surrounding lipids easily.

Cholesterol is a major component of plasma membrane, and it was found that both depletion and enrichment of membrane cholesterol can affect HERG K^+ current. Depletion of membrane cholesterol via sterol binding agent cyclodextrin can shift voltage dependency of activation and deactivation of HERG currents[42]. Enrichment of membrane cholesterol decreases HERG current amplitude[43].

Phosphatidyl inositol bisphosphate (PIP2) is another important lipid which can be modulated during autonomic stimulation. PIP2 can increase HERG current amplitude and hyperpolarizing shift the voltage dependency of activation, accelerate activation and decelerate inactivation[44]. The binding sites for PIP2 are suggested to be located at the C-terminal region of HERG channel (amino acids 883-894) where has higher concentration of positively charged amino acids [45].

Other lipids also affect HERG channel function. Ceramide, a sphingolipid formed during cellular stresses, can reversibly inhibit HERG currents, shift voltage dependency of activation, and accelerate deactivation, probably via facilitation of HERG channel

translocation into lipid raft[46]. Phospholipid metabolite 1-palmitoyl-lysophosphatidylcholine, which accumulated in the ischemia heart, can enhance HERG currents via affecting activation and inactivation[47]. Polyunsaturated fatty acids can induce a use-dependent inhibition of HERG channels with changes on channel activation and inactivation [48].

1.3.4 Potential Interaction Proteins

HERG protein is the pore-forming subunit for Ikr, and the accessory subunit is not clear yet. Several proteins were reported to have the ability to bind HERG channel and modulate HERG K^+ currents.

Mink: Mink was first reported to be associated with KCNQ1 to produce Iks[49]. Later McDonald and colleagues demonstrated that mink can bind HERG protein to form stable complex, and decrease current amplitude with unknown mechanism[50].

MinK-related peptide 1 (MiRP1): It is suggested that MiRP1 which modulates HERG channel gating and pharmacological characterization is a beta subunit of Ikr[51]. The authors demonstrated that MiRP1 can form stable MiRP1/HERG complex, and the recording currents showed decreased peak current amplitude, depolarizing shift of activation and acceleration of deactivation, in the Xenopus oocytes expression system. However, later in mammalian expression system, Weerapura and colleagues demonstrated that MiRP1 may not be an essential part for HERG protein to form Ikr[52].

14-3-3ε: Kagan and colleagues demonstrated that 14-3-3ε can bind HERG channels which are phosphorylated by PKA, and accelerate HERG current kinectics[53].

1.3.5 Phosphorylation by Protein kinases

Phosphorylation on HERG protein by protein kinases can introduce negative charges into channel domain and affect HERG K^+ channel gating. Based on computational prediction and mutagenesis studies, the phosphorylation sites of protein kinase A (PKA), protein kinase C (PKC) and tyrosine kinase in HERG protein have been uncovered [54-56].

PKA: Activation of PKA can reduce HERG K^+ current amplitude, depolarizing shift voltage dependency of activation, accelerate deactivation [56-57]. This modulation relies on direct phosphorylation on HERG protein as the deletion of four potential phosphorylation sites (one located in the N-terminal region and three in the C-terminal region) abolishes this effect[56].

PKC: Pioneered studies on the effect of PKC on HERG channel came from the studies on Phorbol 12-myristate 13-acetate (PMA), a classical but non-specific PKC activator. PMA can reduce HERG current amplitude and change HERG channel gating significantly: deceleration of activation, acceleration of deactivation, depolarizing shift of voltage dependency of activation [58]. However, Kiehn and colleagues demonstrated that the modulation effect of PMA is via PKA[59]. In another study, Cockerill and colleagues reported that direct activation of PKC via 1-stearoyl-2-arachidonyl-glycerol can reduce HERG current amplitude but has no changes on HERG gating[60]. The authors also demonstrated that this modulation relies on the direct phosphorylation on HERG protein within N-terminal region. The discrepancy of these experiments may result from the cross-talk between different protein kinase systems.

Tyrosine kinase: Activating Src tyrosine kinase can increase ERG current amplitude, shift voltage dependency of activation, and decelerate deactivation[61]. Inhibition of tyrosine kinase via inhibitors or inhibiting peptide can decrease ERG current amplitude [54, 61]. The modulation

of tyrosine kinase can be significantly attenuated in HERG mutants Y475A and Y611A, indicates that Y475 and Y611 are the direct phosphorylation sites for this effect [54].

1.3.6 Temperature

Temperature can affect both the voltage-dependency and the rate of HERG channel gating. Higher temperature can induce hyperpolarizing shift in the voltage dependency of activation and depolarizing shift in the voltage dependency of inactivation[22]. Higher temperature can also accelerate activation, deactivation and inactivation [22, 62].

1.4 HERG gene transcription and translation

The transcription of HERG gene is tissue specific. In rat, erg mRNA is abundant in the heart, brain, retina, thymus, and adrenal gland, and is low in skeletal muscle, lung and cornea[63]. The erg mRNA is expressed abundantly and uniformly throughout the heart in human, guinea pig, rabbit, dog and rat[63].

The transcription of HERG gene is also developmentally regulated. The detailed information of this process is mainly from mouse. Maternal ERG mRNA can be detected from oocyte to 2-cell stage. Then ERG mRNA level drops till the 8-cell stage, and rises again in the morula. During this period, the transcription is uneven as inner cell mass cells have higher transcription levels than trophoblast[64]. The expression of ERG proteins throughout preimplantation seems playing a role in controlling the timing of embryo development [65]. From embryonic day 9.5 (E9.5), mouse ERG mRNA can be detected in the heart and the central nervous system (CNS). Later in E13.5, ERG mRNA can also be detected in the peripheral nervous systems, the retina, skeletal muscles, gonads and gut. In the adult mouse, ERG is expressed in the heart, CNS, retina and dorsal root ganglia [66]. Similarly, the transcription of ERG in the skeletal muscles and dorsal root ganglia is limited to the

embryonic phase of development in quail. However, the molecular mechanism of these developmental changes of ERG transcription, especially the transient transcription in certain organs, is not clear.

The transcription of ERG can also be up-regulated in cancer cells and tumors [67-70] . Lin and colleagues studied the upstream of the transcription start site and found several binding sites for oncogenes and tumor suppressor genes. This study indicates that the transcription of HERG channel may be modulated by oncogenes/tumor suppressor genes, which may explain the unusual expression of HERG channels in tumors but not normal original tissues[69].

The transcription and translation of HERG can also be affected in certain pathological situations. For example, persistent atrial fibrillation can decrease the mRNA levels of several potassium channels including HERG channel[71]. Hypoxia can enhance the production of reactive oxygen species in the mitochondria, which decreases HERG translation and current density[72].

Around 25% of reported HERG mutations have premature termination codons (http://www.fsm.it/cardmoc/). Such mutation would give truncated proteins if translated successfully. However, cells have developed nonsense-mediated mRNA decay (NMD) to eliminate the mRNA with premature termination codons. The mRNAs of HERG mutants with premature termination codons, as Q1070X, R1014X and W1001X, can be destroyed by NMD [73-74]. However, it is not clear whether this can be applied to all HERG mutants with premature termination codons.

MicroRNA (miRNA) mediated mRNA silencing is another mechanism to modulate mRNA levels. Xiao and colleagues demonstrated that the up-regulation of miRNA-133 in diabetic patient hearts can decrease HERG current via miRNA mediated mRNA silencing[75].

HERG channel translation can be up-regulated via direct phosphorylation on HERG proteins by protein kinase A[76]. The authors suggest that the phosphorylation of the core-glycosylated form of HERG protein may promote channel folding, and facilitate the whole process of protein translation.

1.5 HERG Channel Assembly

HERG channel consists of four subunits, and the assembly occurred in the ER[77]. In Shaker Kv channels, homo-oligomerization is facilitated by the association of homologous tetramerizing interaction domains[78]. However, similar domain is not found in HERG channel yet. Some reports demonstrated that the N-terminal region of HERG protein is important for channel assembly [77, 79].While other reports show that N-terminal truncations of HERG that lack this domain have the ability to form functional channels [20, 80], which indicates that the assembly signal is not located in this region.

On the other hand, Janke and colleagues reported that tetramerizing coiled-coil domain located in HERG C-terminal regions is involved in channel assembly[81]. This is also supported by the observations that HERG mutants Q725X and p.R744fs failed in oligomeric assembly[82-83].

1.6 HERG trafficking (forward trafficking)

The trafficking of HERG channel is tightly controlled to ensure insertion of properly folded and assembled channels into the plasma membrane. Several factors related to HERG

trafficking have been identified, which include glycosylation, ER retention signal and ER exit signal, molecular chaperones and small GTPases.

1.6.1 Glycosylation

There are two crucial steps for the glycosylation of HERG channel protein. In the ER, high-mannose oligosaccharides are added onto newly synthesized HERG channel and the molecular weight increased from 132 kDa to 135 kDa, as revealed by the treatment of PNGase which can cleave asparagine-linked (N-linked) oligosaccharides from glycoproteins [84]. Other modifications may also occurred in the ER as the size of the deglycosylated HERG is still larger than predicted value 128 kDa from the mRNA sequence. In the Golgi complex, HERG channels undergo further glycosylation process and then translocate into the plasma membrane. The size of the fully glycosylated form of HERG channel protein is about 155 kDa. As this high molecular weight band represents channels located in the Golgi complex and plasma membrane, the density of this band on the Western blot gel is widely used as an important parameter to study HERG channel trafficking. There are two N-linked glycosylation consensus sites (N-X-T/S) in the extracellular S5-S6 linker (N598 and N628)[85]. Only N598 is demonstrated to be the site of core-glycosylation[23]. The non-glycosylatable mutant HERG N598Q channel can traffic normally and produce similar currents as WT channel[23]. This data indicate that glycosylation process itself may not be a crucial step to control the assembly and trafficking of HERG channel.

1.6.2 ER retention signal and ER exit signal

There are several quality control mechanisms in the ER to prevent misfolded and incomplete assembled proteins exit the ER which will affect normal cell function seriously. One mechanism is that the proper folding of protein can mask ER retention signal and exposure

ER exit signal, which in turn trigger the ER exit. For HERG channel, there is a putative ER retention signal in the C-terminus (^{1005} RGR^{1007})[86]. Under normal situation, the C-terminal region can mask this ER retention signal once the protein is properly folded[86].

1.6.3 Molecular Chaperones

Another quality control mechanism is via molecular chaperones. Cytosolic chaperones, heat shock protein (hsp) 70 and hsp 90, can bind the newly-synthesized form of HERG channel and form a transient complex[87]. Hsp 70 can hold the newly-synthesized chain and prevent intramolecular misfolding/aggregation[88] and hsp90 can bind protein to form complex and facilitate channel folding[89]. It is suggested that the formation of transient HERG-hsp70-hsp90 complex is required for proper folding of HERG channel in the ER[90], and the dissociation of this complex is important for channel ER exit[87]. However the exact molecular mechanism for the formation and modulation of this complex is unknown.

ER chaperone protein calnexin can bind immature HERG protein, and this binding requires N-linked glycosyation [84]. It is suggested that this transient binding may modulate HERG channel trafficking.

Hsp40 type 1 chaperones DJA1 (DNAJA1/Hdj2) and DJA2 (DNAJA2) can also affect HERG trafficking via modulate the association of HERG channel and chaperone Heat-shock cognate protein 70 (hsc70), which can go proper folding with hsp90 and Hop and FKBP38, or go degradation way[91-92].

1.6.4 Small GTPase

Movement of proteins between different membrane-bound cellular organelles, such as the ER, the Golgi complex, and the plasma membrane, is regulated by small GTPase. Newly

synthesized proteins can be exported from the ER via COPI or COPII vesicles, which can be regulated by GTPase ARF1 and Sar1 respectively. Delisle and colleagues demonstrated that the impairment of Sar1 but not ARF1 can dramatically block HERG channel trafficking[93]. On the other hand, impairment of small GTPase Rab11B, which regulates the endosomal recycling, also blocks HERG channel trafficking significantly[93]. Taken together, these data indicate that HERG channel may export from the ER via COPII vesicles and endosomal recycling prior to being processed in the Golgi complex.

1.7 Defected HERG channel trafficking

Recent data indicate that the defected trafficking of HERG channels caused either by HERG gene mutations or by drugs can significantly affect HERG function and cause LQTS. The studies carried on the mechanisms of HERG trafficking defect and the restoration of normal trafficking will be beneficial for therapeutic purpose.

1.7.1 The mechanisms of HERG trafficking defects

HERG mutations induced ER retention: Protein trafficking defects are the most common mechanism for LQT2 HERG mutations [27, 37]. The mutation sites in these trafficking defected HERG genes are mainly located in putative alpha-helical or beta-sheet domains, which including Per-ARNT-Sim (PAS) domain, transmembrane domain, S5-P linker, pore loop and cyclic nucleotide binding domain (cNBD) [27, 37]. On the other hand, the mutants out of these domains are not likely to cause trafficking defect. Taken together, these data further emphasize that the proper folding of HERG channel is crucial for its normal trafficking.

However, how cells detect the misfolding of HERG channel is not clear. There is one report showing that the interactions between the mutant HERG channel and hsp70/hsp90 are prolonged compared with the WT HERG channel[90]. This prolonged association has been

interpreted as the attempt to hold the misfolded mutant HERG protein in the ER by hsp70, and re-fold them into correct conformation by hsp90. Another report also indicates that the binding between the channel and chaperone molecule calnexin is prolonged for HERG mutant N470D compared with WT HERG [84]. The mutations located in the PAS domain and cNBD domain (exposed to the cytosol) may directly interfere the binding with cytosol molecular chaperones as hsp70 and hsp90.

In heterozygous patients, the trafficking defected HERG mutants may also affect WT HERG channel trafficking. It is reported that the trafficking defect HERG A651V assembled with WT HERG protein and retained most heterotetrameric channels in the ER[94].

HERG mutants affect the interaction between HERG channel and GM130 in the Golgi complex. The physiological role for the interaction between HERG channel and GM130 is not clear. However, HERG mutants (S818P, V822M and R823W) located in the cNBD domain can disrupt the interaction between HERG protein and GM130 by a yeast two-hybrid assay[95]. This disruption may block the intra-Golgi trafficking of mutant proteins. However, more studies are needed to clarify the molecular mechanism and the physiological role of this binding.

Drug induced trafficking defect

Drug induced HERG trafficking defect is a new mechanism for the acquired LQTS. Surprisingly, almost 40% of HERG channel blockers have the ability to induce HERG trafficking defect [96]. Further, drugs which do not block HERG channel also impair channel trafficking[96] . Despite the number of the drugs impair HERG trafficking, less is known about

the molecular mechanism except two groups of drugs: hsp90 inhibitors and Na^+/K^+ ATPase blockers.

Hsp90 inhibitors: As mentioned previously, hsp90 is an important cytosolic chaperone assisting HERG channel folding in the ER. It is no doubt that the inhibition of hsp90 function may affect the maturation of HERG channel and promote channel degradation in the ER [87]. Further, Ficker and colleagues suggested that not only specific hsp90 inhibitors, but also other cardiotoxic compounds such as arsenic trioxide and potassium antimony(III) tartrate may impair HERG channel trafficking via the inhibition of hsp90 function [97].

Na^+/K^+ ATPase blockers: It is well known that Na^+/K^+ ATPase plays important roles in maintaining the Na^+/K^+ gradient crossing the plasma membrane. Recent studies show that the depletion of intracellular K^+ by the blocking of Na^+/K^+ ATPase may impair the folding of HERG protein in the ER, which retains the impaired channel in the ER [98-99].

1.7.2 Restoration of normal HERG trafficking

The studies on the restoration of normal HERG trafficking may uncover new therapeutic applications for LQTS patients. Till now there are three ways to restore normal HERG trafficking: Lowing the temperature, non-specific chemical chaperones and specific pharmacological chaperones. Anderson and colleagues reported that 19 out of 28 (68%) HERG trafficking defect mutants can be rescued and different mutants can be rescued with different conditions[27].

Lowing the temperature: Culture temperature is very important for HERG channel trafficking. Chen and colleagues demonstrated that the temperature between 27 and 30 degree is the optimum condition for WT HERG channel trafficking in CHO cells[100].

Decreasing culture temperature can successfully restore the majority of HERG trafficking defects caused by HERG mutations[27] or drugs [99]. This restoration can be verified by recording HERG currents via patch clamp recording, or by detecting fully-glycosylated form of HERG protein on Western blot gel, or by immunostaining. There are three possible mechanisms for the effect of temperature on HERG trafficking. The first mechanism is that lower temperature can prolong the interaction between the HERG channel and chaperones, and increase the chance of obtaining the proper folding conformation. The second mechanism is that less HERG proteins will be degraded at lower temperature, and more HERG channels can reach the plasma membrane. The third mechanism is that at normal culturing temperature of 37 degree, the majority of HERG protein may be rapidly internalized after inserted into the plasma membrane. Lower temperature can reduce this process and more functional channels can stay longer on the plasma membrane[100].

Non-specific chemical chaperones: Small molecule chemical chaperones such as glycerol and DMSO are believed have the ability to stabilize protein conformation during the maturation process[101]. Glycerol can rescue trafficking defect mutant HERG N470D [102]. However, the super high concentrations needed for chemical chaperones to restore HERG protein trafficking strongly restrict their clinical use.

Thapsigargin, a sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitor, can rescue HERG trafficking defect induced by gene mutation [27, 103]. However this effect is not likely via the inhibition of SERCA function as other SERCA inhibitors DBHQ and cyclopiazonic acid failed to rescue HERG trafficking defect. The mechanism is still unknown.

Specific pharmacological chaperones: HERG channel blockers can rescue most HERG trafficking defects caused by mutations[27], and the efficacy for channel rescue is directly linked to the potency of channel blocking [104]. It is suggested that HERG channel blockers can bind and stabilize channels, which in turn facilitates the trafficking of mutated channels [104].

Interestingly, pharmacological chaperones can also restore drug induced HERG trafficking defect. For example, HERG channel blocker atemizole can restore the trafficking impaired by cardiac glycosides, probably via stabilize the affected HERG channel[98].

1.8 Endocytosis (backward trafficking)

The density of proteins in the plasma membrane is dependent on the equilibrium between exocytosis (forward trafficking) and endocytosis (backward trafficking). However, the studies on the backward trafficking of HERG channels are rare. The available data indicate that the internalized HERG channels can either enter endosomes and recycle back to the plasma membrane, or fuse into lysosomes for degradation. Diacylglycerol can trigger protein kinase C evoked endocytosis of HERG channels[105]. These internalized HERG channels are located in the early endosomes but not lysosomes. On the other hand, lower extracellular potassium or ceramide can accelerate HERG channel internalization and the affected HERG channels can be infused into lysosome [106-107].

1.9 HERG channel degradation

WT HERG channels have a half life time around 8-12 hours, and mutant HERG channels have a short life time[84 107]. The life time of HERG channel protein is mainly determined by protein degradation, either proteasomal degradation or lysosomal degradation.

The proteasome degradation usually works on the newly synthesized HERG proteins which failed in passing the quality check in the ER. Trimming of mannose and adding polyubiquitin onto the core-glycosylated HERG channel are necessary for proteasomal degradation of HERG proteins [91, 108]. Polyubiquitination of HERG protein needs E3 ubiquitin ligase, C-terminus of Hsc70-interacting protein[91]. However, the mechanism on the detecting and marking misfolded HERG channel for proteasomal degradation is not clear.

On the other hand, lysosomal degradation can work on the plasma membrane HERG proteins which are internalized into endosomes and fused into lysosome later. The knowledge on the lysosomal degradation of HERG channel during normal situation is lacking. The only information is from the studies on the enhancement of lysosome degradation of HERG channels induced by ceramide and depletion of extracellular potassium [28, 106-107, 109]. The internalized HERG channels traffic from endosomes to multivesicular bodies and finally lysosomes. Monoubquitination but not polyubiquitination, is the sorting signal for the lysosome degradation of HERG channels.

1.10 Current progress on the modulation of sex hormones on HERG K^+ channels

Sex hormones include androgen, estrogen and progesterone. Androgen level is high in male and considered as “male sex hormone”. Estrogen and progesterone levels are high in female and considered as “female sex hormones”. Sex hormones have a steroid nucleus composed of four fused rings. All of these hormones can enter the cells easily, bind a variety of targets, and execute multiple physiological functions. Heart is one of the target organs for sex hormones and increasing data reveal the impacts of gender and sex hormones on human cardiac rhythm and arrhythmias. For example, Proarrhythmic drugs induce torsades de

pointes (TdP) arrhythmia more frequently in women than men[110]; women have longer heart rate-corrected QT intervals than men[110]; QTc intervals of males shorten after puberty[110]. It is believed that sex hormones are involved in these gender and development-related changes and great efforts have been put to study the effects of sex hormones on cardiac ion channels.

Sex hormones as estrogen and androgen at super high concentration can rapidly inhibit various cardiac ion currents, including L-type Ca^{2+} currents ($I_{Ca,L}$), T-type Ca^{2+} currents ($I_{Ca,T}$), slowly- and rapidly-activating delayed rectifier K^+ currents (I_{ks}, I_{kr}), transient outward current (I_{to}), and inwardly rectifying K^+ current (see reviewed[111]). At physiological levels, androgen and progesterone can potentiate I_{ks}, and attenuate $I_{Ca,L}$ [112-113]. On the other hand, chronic androgen treatment can increase the protein expression level and single-channel activity of Ca^{2+} channel[114].

HERG K^+ channel can also be modulated by sex hormones. Estrogen at 30 μmol/L can rapidly inhibit I_{kr} [115]. While at the physiological range, both testosterone and progesterone has no acute effect on I_{kr} [112-113]. However, chronic treatment with androgen increases cardiac I_{kr} in a rabbit model, which is correlated to the gender difference of QT interval and cardiac I_{kr} density, although the potential molecular mechanisms are unknown[116-117]. These reports indicate that HERG K^+ channel may be an important target for sex hormone and the modulation would explain the gender and development related arrhythmia. The chronic effects of sex hormones on HERG K^+ channel are important as the levels of sex hormones are relatively stable [118-119].

1.11 Purpose of this study

The purpose of this thesis is to systematically investigate the effect of sex hormones on HERG K^+ channel expression, trafficking and function. Specifically, current study sought to:

I. screen sex hormones in a heterologous expression system and find out which hormone(s) may affect HERG K^+ channel function. Western blotting analysis and patch clamp will be used to study HERG channel expression and function.

II. investigate the effect of sex hormones on biogenesis of HERG K^+ channel function. RT-PCR, Western blotting, and pharmacological manipulation will be employed to study the transcriptional and translational mechanisms for the effect of sex hormones on HERG channel expression.

III. examine the effect of sex hormones on trafficking of HERG K^+ channel function. Western blotting, confocal microscopy, biological assay and pharmacological manipulation will be employed to study HERG trafficking.

IV. investigate the effect of sex hormones on protein degradation of HERG K+ channels. Western blotting, biological assay and pharmacological manipulation will be employed to study HERG protein degradation.

V. study the effect of sex hormones on ERG K^+ channel in cardiac tissues. Rabbit or neonatal rat cardiac myocytes will be used to study the effect of sex hormones on the I_{kr} current and ERG K^+ channel protein.

Chapter 2 Stimulation of androgen receptor AR45 variant stabilizes HERG potassium channel protein via activation of extracellular signal regulated kinase 1/2

This part of work has been published in:

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2.1 Introduction

Proarrhythmic drugs induce *torsade de pointes* (TdP), a life-threatening arrhythmia, more frequently in women than men [120]. The slower rates of cardiac repolarization and the longer QT intervals in women compared to men are suspected to be responsible for the greater risk in women [121-122]. Latest clinical and basic studies suggest that androgens may be a key factor for the sex difference in heart rate-corrected QT interval (QTc). The QTc intervals were shortest in post-pubescent males who have higher androgen concentration, in normal populations and in families with inherited long QT syndrome (LQTS)[122-123]. Cardiac repolarization periods in castrated men and normal women who have lower androgen concentration were longer than those in normal men and women with virilization who have higher androgen concentration[124]. Moreover, testosterone replacement therapy restored male ECG patterns of repolarization in the castrated men[124]. In animal experiments, androgen replacement treatment also restored the prolonged QT intervals in orchiectomized male rabbits [116]. Taken together, these experiments indicate that androgens may play important roles in the gender difference in QTc, which contributes to the higher risk of developing drug-induced TdP in women[120].

HERG K^+ channel is one of the ion channels controlling the repolarization of action potential in cardiac myocytes[125-127]. Malfunction of HERG channel caused by mutations or drugs can prolong QT intervals and induce TdP arrhythmia[125, 128-129]. The finding that I_{Kr} current densities in male rabbit ventricular myocytes are significantly higher than those in the female [117] indicates that the modulation of androgen on ERG/I_{Kr} may play a role in the sex difference of QT intervals. Later, this research group reported that androgen replacement

treatment increased I_{Kr} currents but had no effect on ERG mRNA levels in gonadectomized rabbit hearts, which suggests that androgen modulate I_{Kr} in a post-translation mechanism[116].

It is well established that the heart is a target organ of androgens[130], but the presence of androgen receptor (AR) in the heart is controversial[131-134]. Recently, Ahrens-Fath and colleagues reported that AR transcript levels were very low in human heart compared to those in the liver or testis; however, the transcript level of AR45, a naturally occurring variant of AR, was very high in human heart tissue [135]. This data suggested that in the heart AR45 may be the main receptor for androgen. In this study, we investigated the effect of androgens on HERG protein expression and function in heterological expression system and isolated rabbit cardiac myocytes.

2.2 Materials and Methods

2.2.1 Materials

5β-dihydrotestosterone (5β-DHT) was purchased from Steraloids. R1881 was from NEN. PD98059 and U0126 were from Merck. All other reagents were from Sigma. Testosterone, 5α-dihydrotestosterone (5α-DHT), 5β-DHT, progesterone, estradiol and flutamide were dissolved in ethanol as stock solutions. N-acetyl-L-leucyl-L-leucyl-L-norleucinal (ALLN), cycloheximide (CHX), MG132, R1881 and bafilomycin A1 were dissolved in DMSO as stock solutions. The final ethanol and DMSO concentrations were no more than 0.2%.

2.2.2 Cell culture and transfection

Human embryo kidney (HEK293) and Chinese hamster ovary (CHO) cells are androgen receptor negative cells [136-137]. HERG-HEK293 and CHO cells were cultured in RPMI 1640 (GIBCO, USA) or F-12 (Sigma, USA) cell culture medium, respectively, supplemented with 10% fetal bovine serum (FBS, Hyclone, USA) and penicillin-streptomycin (GIBCO, USA) at

37°C in a 5% CO_2 atmosphere. We established a HEK293 cell line stably expressed HERG K^+ channels, HERG-HEK293. Briefly, HEK293 cells were transfected with HERG-myc plasmid in pCI-Neo vector (Promega, USA). The stable clones were selected by culturing in medium containing G418 (800 μg/ml) and confirmed by Western blot analysis and electrophysiological study. For biochemical analysis, pSG5-AR45, pSG5-AR or GFP were transiently transfected into HERG-HEK293 cells with Lipofectamine2000 (Invitrogen, USA). Briefly, HERG-HEK293 cells were cultured in RPMI 1640 medium supplemented with 3% FBS for 1 day and then were transfected with AR45, AR or GFP and kept in the above culture medium overnight. After serum starving for 16 h in RPMI 1640 medium with 0.5% FBS, cells were treated with androgens. For mechanism studies, inhibitors were added to culture medium 1 hour before and during androgen treatment. For electrophysiological studies, CHO cells were transiently transfected with cDNAs of HERG, AR45 and GFP by electroporation as described previously [44-45]. Briefly, CHO cells were electroporated (single 160 V pulse for 15 ms) with 4 μg HERG, 4 μg AR45 and 2 μg GFP cDNAs in a 2-mm gap cuvette using a Gene Pulser Xcell (BIO-RAD). After electroporation, the cells were plated sparsely and grown on sterile glass coverslips in 60-mm tissue culture dishes. Cells were serum starved following the same protocol as mentioned above in the protocol for biochemical analysis. Forty eight to 72 hours after transfection, cells were used for electrophysiological studies.

2.2.3 Isolation of rabbit cardiac myocytes

Myocytes were isolated from rabbit heart via enzymatic digestion as described previously [44-45]. The heart was quickly excised from anesthetized male rabbits (1 month), and perfused with calcium free Tyrode's solution (at 37^{o}C) containing (in mM) 137 NaCl, 5.4 KCl, 1

MgCl2, 10 HEPES, and 10 glucose. Then, the perfusion solution was changed to the Tyrode's solution containing 1 mg/ml collagenase (Worthington type II) and 0.28 mg/ml protease (type XIV, Sigma) and perfused for 20-25 minutes. The ventricular tissue was then cut into small pieces filtered through a 250 μm mesh screen. The cells were washed and the calcium concentration of Tyrode's solution was adjusted to 1.25 mM gradually. Isolated myocytes were resuspended in M199 medium and plated into laminin (Sigma)-coated Petri dishes. Cells were treated with androgens with the same protocols as described above.

2.2.4 Western blotting analysis

Cells were washed with ice-cold PBS and then ice cold lysis buffer (150mM NaCl, 25mM Tris-HCl pH 7.5, 5mM EDTA, 1% NP-40, 0.4% deoxycholic acid and EDTA-free protease inhibitor cocktail tablets (Roche Pharmaceuticals)) was added. Lysates were rocked for 1 hour at 4 °C and then centrifuged at 14,000 × *g* for 10 min at 4 °C. The supernatants were assayed for total protein content (Bio-Rad Protein Assay), and equal amounts (50-100 μg) of cell lysate protein were subjected to SDS-PAGE analysis. Protein samples were combined with 4 × SDS-PAGE sample buffer (4% (w/v) SDS, 40% glycerol, 20% (v/v) β-mercaptoethanol, 0.004% (w/v) bromphenol blue, 125 mM Tris buffer, pH 6.8) incubated for 5 min at room temperature, separated on a 8-10% SDS-PAGE, and electrophoretically transferred onto 0.2 μM nitrocellulose membrane (Bio-Rad Laboratories). Membranes were blocked in 10% non-fat dry milk and 0.1% Tween 20 in TBS for 1 h at room temperature, and incubated with appropriate primary antibodies (1:250 to 1:1000 dilutions in 5% bovine serum albumin (BSA, Sigma) and 0.1% Tween 20 in TBS overnight at 4 °C. The membranes were then washed with 0.1% Tween 20 in TBS and incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (1:10,000 to 1:50,000 dilution) for 1 h at room

temperature. After 3 washes as above, horseradish peroxidase-bound protein was detected by chemiluminescence (Pierce, SuperSignal West Pico Chemiluminescent Substrate). Anti-myc mouse monoclonal 9E10 and anti-tubulin rabbit monoclonal antibodies were from Sigma. Anti-phosphorylated extracellular signaling-regulated kinase 1/2 (ERK1/2) antibody was from Cell Signaling. Anti-Kv11.1 (HERG)-Extracellular antibody was from Alomone Labs. Other antibodies were from Santa Cruz Biotechnology, Inc. All gels illustrated in the figures are representative examples from 4 to 8 independent experiments. ß-tubulin was applied as an internal control to normalize protein loading. The intensity of bands was quantified using LabWorks^{TM} Image Analysis software (UVP).

2.2.5 Cycloheximide-based protein chase experiment

After posttransfection for 12 h, the medium was replaced with fresh RPMI 1640 plus 0.5% FBS with/without 5α-DHT. Eight hours later, cycloheximide (40 μmol/L) was added into medium to stop protein synthesis. Cells were then harvested at different time points (0, 6, 12 and 24 h) after cycloheximide treatment. Total cell lysates were analyzed by Western blotting as described above. The amount of HERG remaining at each time point was normalized with the original HERG amount at time zero. Protein degradation rate is expressed as half-life ($t_{1/2}$), the time for degradation of 50% of the protein.

2.2.6 RT-PCR

Cells were rinsed twice in cold sterile PBS and total RNA was extracted by RNeasy Kits (Qiagen) following manufacturer's instruction. Total RNA was quantified by spectrophotometric analysis and checked for purity and quality by running a small aliquot on a 1% agarose gel. RT-PCR was carried out using QIAGEN® one-step RT-PCR kit (Qiagen). The following primers were used to examine the mRNA expression of HERG: forward: 1999

5’-TCCAGCGGCTGTACTCGGGC-3’, reverse: 2573 3’-GGACCAGAAGTGGTCGGAGAACTC-5’. As a quality control, PCR amplification of the messenger relative to the housekeeping gene GAPDH was performed using the following primer pair: forward: 1644 5’-TGATGACATCAAGAAGGTGGTGAAG-3’; reverse: 1883 5’-TCCTTGGAGGCCATGTAGGCCAT-3’. Reverse transcription was performed at 50°C for 30 min. PCR protocols consisted of a 15 min initial activation step at 95°C, followed by 25 cycles of amplification. Each cycle included 1 min at 94°C, 1 min at 55°C and 1 min extension at 72°C (30 s each step for GAPDH). The reactions were terminated by an elongation step at 72°C for 10 min. PCR products were analyzed with 1% agarose gel electrophoresis and imaged by the Multigenius Bioimaging system (Syngene, UK).

2.2.7 Confocal microscopy

HERG-HEK293 cells seeded onto glass coverslips were subject to formaldehyde fixation before indirect immunofluorescence staining. Primary antibodies were detected with Alexa Fluor 568–conjugated anti-goat Ig and Alexa Fluor 488–conjugated anti-rabbit Ig (WGA; Invitrogen). After staining, coverslips were mounted with Prolong Gold antifade reagent (Invitrogen), and confocal images were acquired using Leica TCS SP5 confocal system. Alexa-488 images were acquired with excitation at 488 nm using an argon laser. Alexa-568 images were acquired with excitation at 543 nm using a helium neon laser. Dual-color analysis was done with appropriate single-color controls with individual channels recorded sequentially. Instrument settings were adjusted to obtain minimal saturated pixels, and where semiquantitative comparisons were done, the settings were kept constant. Negative controls include slides probed with either primary antibody or secondary antibody alone.

2.2.8 Patch Clamp recording

Cells on cover slips were taken directly from the cell culture incubator and placed in an acrylic/polystyrene perfusion chamber (Warner Instruments) for electrophysiological measurements. Patch pipettes were pulled and polished to obtain a tip resistance of 2-3 MΩ in the patch clamp solutions. For whole-cell voltage-clamp the pipette solution consisted of (in mM) KCl 126, $MgSO_4$ 2, $CaCl_2$ 0.5, EGTA 5, Mg-ATP 4, and HEPES 25 (pH7.2, osmolality: 280±10 mOsm). External bath solution consisted of (in mM) NaCl 150, $CaCl_2$ 1.8, KCl 4, $MgCl_2$ 1, glucose 5, and HEPES 10 (pH7.4; osmolality: 320±10 mOsm). All experiments were carried out at room temperature (20-22º C). Cells were studied on an inverted microscope (Nikon) equipped with electronic patch-pipette micromanipulators (Burleigh) and epifluorescence optics for GFP transfected cells. An Axopatch-200B patch clamp amplifier (Axon Instruments) was used for voltage-clamp measurements. The series resistances were about 9 to 10 MΩ. Voltage-clamp protocols were controlled using pClamp9.2 acquisition and analysis software. To elicit HERG K^+ currents depolarizing voltage pulses were applied to various levels from a holding potential of –70 mV for 4 s followed by stepwise repolarization to –70 mV to measure outward tail currents. Signals were analog-filtered at 2,000 Hz and sampled at 5,000 Hz. Voltage-dependent activation data were fitted to a Boltzmann equation: $I = 1/(1+\exp[(Vh-V)/k])$, where I is the relative tail current amplitude, V is the applied membrane voltage, Vh is the voltage at half-maximal activation, and k is the slope factor.

2.2.9 Statistics

Values presented are means ± SE. ANOVA was employed for statistical analysis of the data and P-values of < 0.05 were considered to be significant.

2.3 Results

2.3.1 Androgen up-regulated expression of HERG protein via AR45

Firstly, we examined the effects of androgen on HERG protein expression in the presence and absence of androgen receptors (AR and AR45). 5α-DHT, a potent and non-aromatisable androgen, increased both mature (~155 kDa) and immature (~135 kDa) forms of HERG protein significantly in HERG-HEK293 cells transfected with AR45, but did not change HERG protein expression in HERG-HEK293 cells transfected with AR or GFP plasmids (Figure 2-1 A). Similar results were also observed in HEK293 cells that transiently co-transfected HERG cDNA with AR45, AR or GFP cDNAs (data not shown). These data suggest that 5α-DHT may increase HERG protein abundance via stimulating AR45, but not AR. Interestingly, AR but not AR45 increased HERG protein level in a hormone-independent manner. This upregulation may arise from intrinsic hormone-independent activity of AR[138]. For the following studies, we studied HERG-HEK293 cells transfected with AR45 plasmids, unless otherwise stated.

Then we studied the concentration-dependent effect of androgen on HERG protein. Figure 2-1 B shows that 5α-DHT increased HERG protein levels in a dose-dependent manner, and the concentration of half maximal effect (EC50) is 1.04 ± 0.54 nmol/L. The EC50 of 5α-DHT is within the physiological concentration range in men but not women. As it is reported that the serum 5α-DHT level is 1.5±0.8 nmol/L in men [119] and around 0.02 nmol/L in women[139]. The maximum effect was observed when using 10 nmol/L of 5α-DHT, and this concentration (10 nmol/L) was chosen for the other experiments.

We also tested other three androgens (testosterone, 5β-DHT, and R1881) and found that all of them significantly increased HERG abundance (Figure 2-1 C).

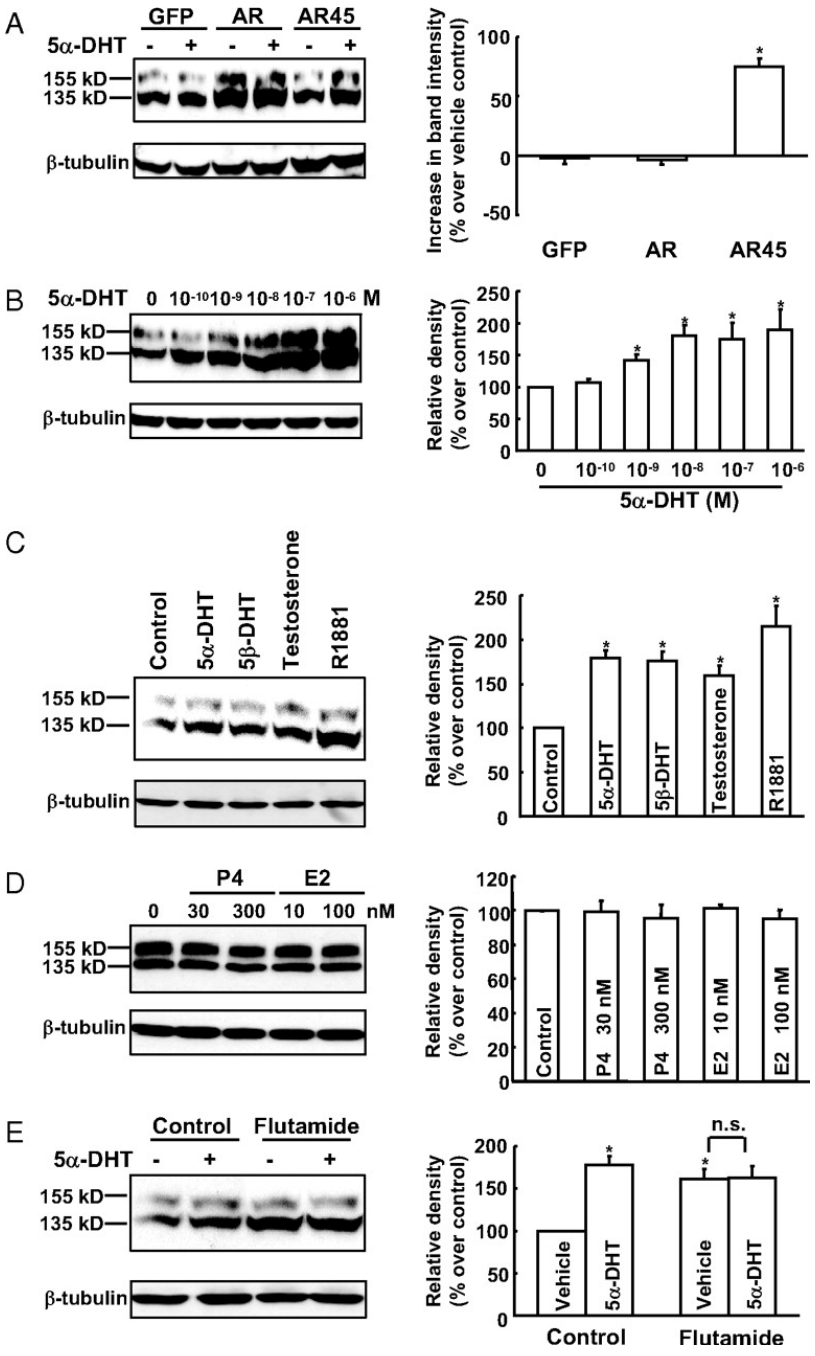

Figure 2-1 Effect of androgens on HERG protein in HERG-HEK293 cells transfected with GFP, AR or AR45. (A) 5α-DHT treatment (10 nmol/L, 24 h) upregulated HERG protein expression in HERG-HEK293 cells transfected with AR45, but not in the cells transfected with GFP or AR plasmids. (n = 6 to 8 per group). (B) Concentration-dependent effect of 5α-DHT in HERG-HEK293 cells transfected with AR45. Western blot analysis indicated that treatment with 5α-DHT (10^{-10}-10^{-6} M) for 24 h increased HERG protein level in a concentration-dependent manner. (n = 6 to 7 per group). (C) Treatment with 5α-DHT (10 nmol/L), 5β-DHT (10 nmol/L), testosterone (100 nmol/L) or R1881 (10 nmol/L) for 24 h significantly increased HERG protein

abundance. (n = 4 to 8 per group). (D) Treatment with progesterone (P4, 30 and 300 nmol/L) or estradiol (E2, 10 and 100 nmol/L) for 24 h failed to affect HERG protein abundance. (n = 6 per group). (E) The effect of androgen (10 nmol/L 5α-DHT) and antiandrogen (10 μmol/L flutamide) on HERG protein levels (n = 8 per group). Representative Western blots are shown in the left panels. The percentage increment in protein density on treatment with 5α-DHT is shown in the right panels. HERG protein abundance was normalized to β-tubulin. Data are means ± S.E. *, $p<0.05$.

On the other hand, other sex hormones as progesterone (30 and 300 nmol/L) and 17-β estradiol (10 and 100 nmol/L) did not change HERG protein level (Figure 2-1 D). These data suggest that androgens stimulate AR45 and up-regulate HERG protein expression specifically.

To further confirm the involvement of androgen receptor, flutamide, an antiandrogen, was used. It was found that flutamide (10 μmol/L) attenuated the effect of 5α-DHT (Figure 2-1 E). Interestingly, flutamide itself also increased HERG protein level and this may from the non-genomic androgen-like effects of the drug [140-141].

2.3.2 The effect of 5α-DHT on HERG protein level was mediated via a post-translational mechanism

The increase of HERG channel protein level may be resulted from enhanced protein synthesis or decreased protein degradation. Firstly, we tested whether androgen increased HERG protein level via a genomic effect. The mRNA level of HERG was examined by RT-PCR, and it was found that androgen treatment did not change HERG mRNA level (Figure 2-2 A). We also tested a plasmid with same vector but inserted with kir2.1 gene. However, the stimulation of AR45 by 5α-DHT failed to change kir2.1 protein level (Figure 2-2 B). To test whether augmentation of HERG protein level was due to the change of protein synthesis, cycloheximide, a protein translation inhibitor, was employed. It was found that cycloheximide alone decreased HERG protein levels, and it did not prevent the stimulatory

effect of androgen (Figure 2-2 C). Taken together, these data indicate that a post-translational mechanism is underlying the effect of 5α-DHT.

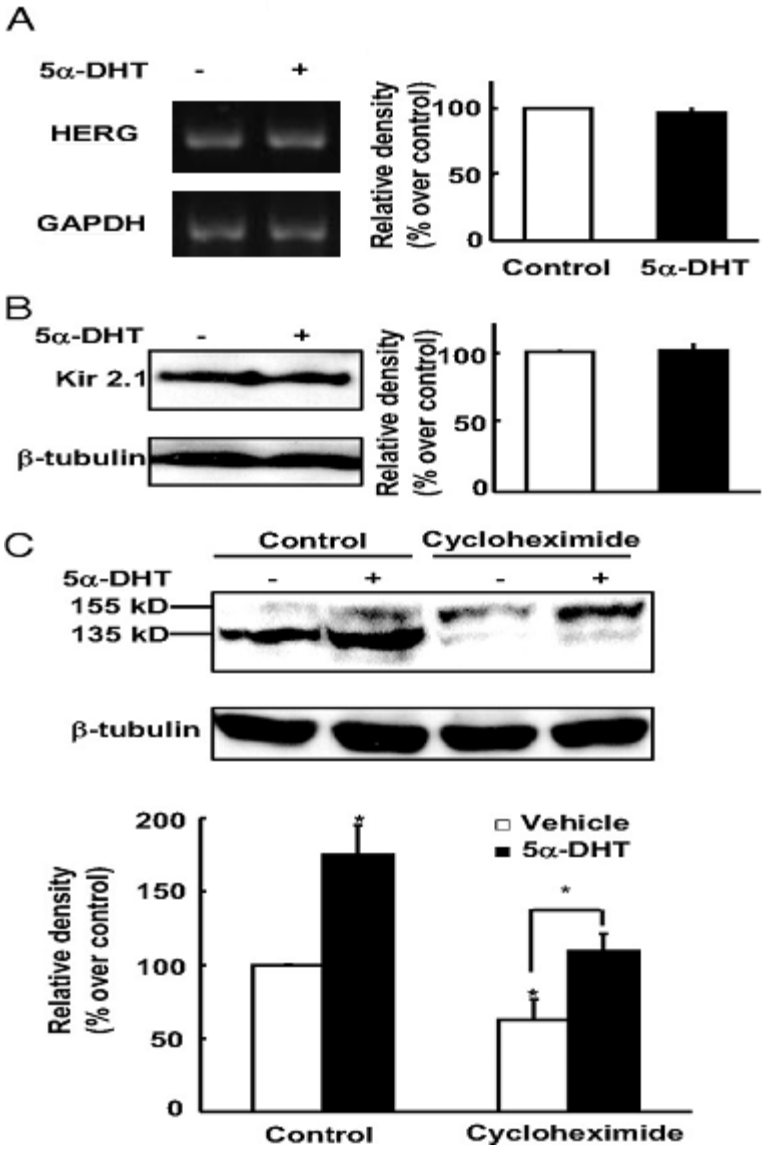

Figure 2-2 Post-translational effect of 5α-DHT on HERG protein levels. (A) RT-PCR for the measurement of HERG mRNA levels with 5α-DHT treatment, the HERG mRNA levels were normalized with GAPDH (n=4). (B) Immunoblot analysis showed that 5α-DHT (10 nmol/L) had no effect on Kir2.1 protein level (n=4). (C) Immunoblot analysis showed that 5α-DHT (10 nmol/L) increased HERG protein levels in the presence or absence of cycloheximide (40 μM). Cycloheximide was applied 1 h prior to administration of 5α-DHT (n = 5 per group). Data are means ± S.E. *, $p<0.05$. Copyright 2008, The Endocrine Society.

2.3.3 5α-DHT increased HERG protein abundance at the cell surface and in the intracellular compartments

To examine the effect of androgen on HERG protein trafficking, we performed double immunofluorescence staining. GM130 was chosen as a marker of the Golgi complex and calnexin as a marker of the endoplasmic reticulum (ER). Chronic androgen treatment increased HERG signals in the plasma membrane, the ER and the Golgi complex, but without clear preferential colocalization with GM130 or calnexin (Figure 2-3). This result indicates that 5α-DHT does not alter HERG channel trafficking.

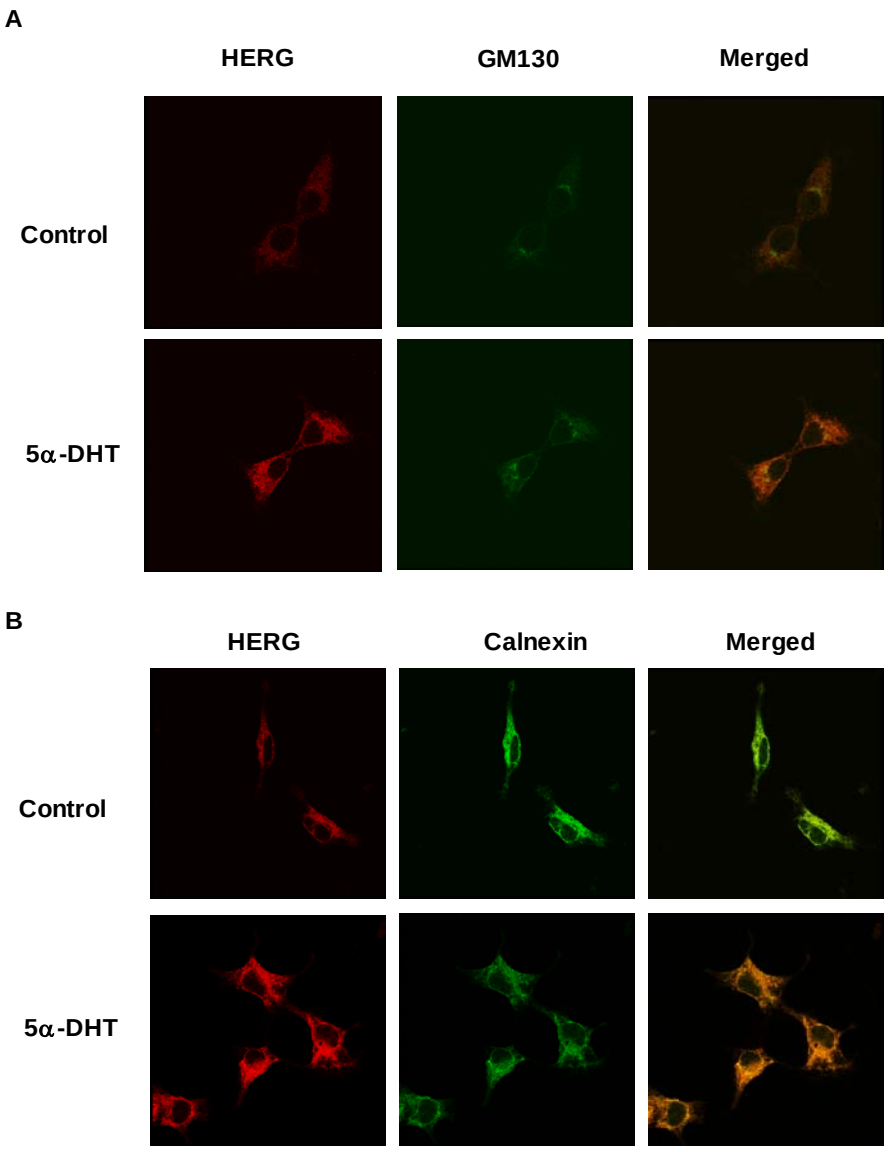

Figure 2-3 Effect of 5α-DHT on subcellular localization of HERG. Immunofluorescence assays with double staining for HERG-*myc* (red channel) and either the Golgi marker GM130 (A) or the ER marker calnexin (B)(green channel). 5α-DHT treatment (10 nmol/L for 24hrs) resulted in globally increased HERG signal in both compartments as well as on the surface. Copyright 2008, The Endocrine Society.

2.3.4 The effect of 5α-DHT on HERG protein in the presence of protein degradation inhibitors

Then we tested the involvement of protein degradation for the effect of 5α-DHT. HERG can be degraded by either lysosomal or proteasomal degradation pathways[106, 108]. In this study, we employed a lysosomal protease inhibitor, bafilomycin A1, and two proteasome inhibitors, MG132 and ALLN. Bafilomycin A1, MG132 and ALLN alone significantly increased HERG protein level, which is in agreement with previous studies that HERG proteins can be degraded by lysosome or proteasome (Figure 2-4). Moreover, these inhibitors significantly abolished the effect of 5α-DHT (Figure 2-4). These data suggest that androgen may inhibit protein degradation.

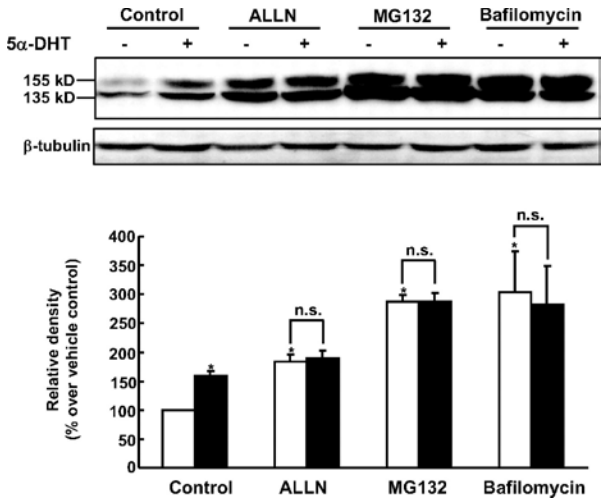

Figure 2-4 Effect of 5α-DHT on HERG protein levels in the presence and absence of proteasome and lysosomal protease inhibitors. Immunoblot analysis shows that the upregulation of HERG protein abundance was abolished by two proteasome inhibitors ALLN (50 μM) and MG132 (50 μM), and a lysosome inhibitor, bafilomycin A1 (100 nmol/L). Inhibitors were applied 1 h prior to administration of 5α-DHT. Data are means ± S.E. n = 5 to 8 per group, *, $p<0.05$. Copyright 2008, The Endocrine Society.

2.3.5 5α-DHT prolonged the half-life of HERG protein

To confirm the above findings, we employed the cycloheximide chase assay to study the turnover rates of HERG protein. It was found that 5α-DHT treatment delayed HERG protein degradation significantly at different time points. The half-life time ($t_{1/2}$) of HERG was prolonged from 12.4 ± 1.5 h (control) to 16.3 ± 1.2 h (5α-DHT treatment). This result suggests that androgen can enhance HERG protein stability.

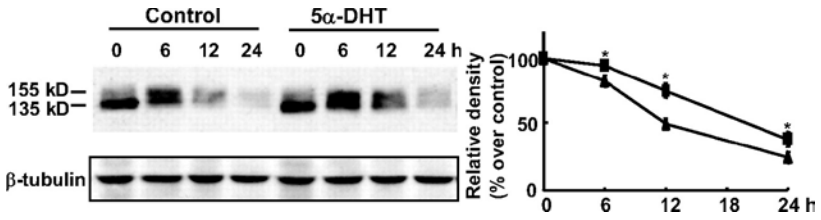

Figure 2-5 Effect of 5α-DHT on HERG protein stability. HERG protein stability was examined with cycloheximide-based chase assay. Immunoblot analysis shows that treatment with 5α-DHT (10 nmol/L) prolonged HERG protein half-life. Data are means ± S.E. n = 5 per group, *, $p<0.05$. Copyright 2008, The Endocrine Society.

2.3.6 The effect of 5α-DHT on HERG protein level in the presence of ERK1/2 inhibitors

Several studies demonstrated that androgens can exert non-genomic effects via ERK1/2 MAPK signaling pathway [142-146]. To examine the involvement of ERK1/2 in the effect of 5α-DHT on HERG protein, we first tested whether 5α-DHT treatment can induce the phosphorylation of ERK1/2. It was found that 5α-DHT induced phosphorylation of ERK1/2 (a marker for ERK 1/2 activation) in a dose-dependent manner, in the HERG-HEK293 cells transfected with AR45 but not in the cells transfected with GFP (Figure 2-6 A). This result indicates that androgen can activate ERK1/2 via AR45. Then we tested whether ERK1/2 inhibitors (PD98059 and U0126) can abolish the effect of androgen on HERG channel. It was found that PD98059 and U0126 did not change HERG protein level, while the two

inhibitors countered the effect of 5α-DHT on HERG protein abundance (Figure 2-6 B). Taken together, these data suggest that the activation of ERK1/2 mediates the increase of HERG channel protein level via activating AR45by androgen.

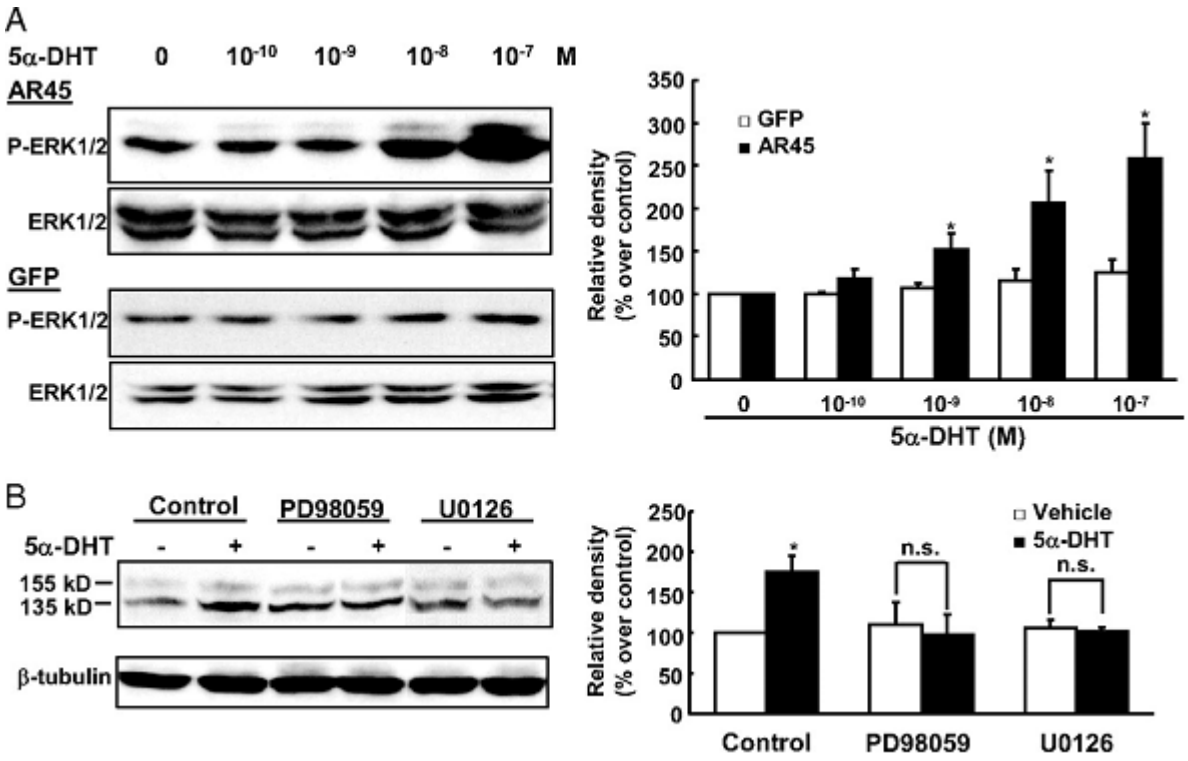

Figure 2-6 Effect of 5α-DHT on HERG protein levels was via activation of ERK1/2. (A) Immunoblot analysis showing that treatment with 5α-DHT (10^{-10}-10^{-7} M) for 30 min concentration-dependently induced phosphorylation of ERK1/2 in cells transfected with AR45 (n = 4), but not in cells transfected with GFP (n=6); (B). Immunoblot analysis showing that two ERK1/2 inhibitors, PD98059 (20 μM) and U0126 (20 μmol/L), abolished the effect of 5α-DHT on HERG protein levels (n = 4 to 7 per group). Inhibitors were applied 1 h prior to administration of 5α-DHT. Data are means ± S.E. *, $p<0.05$. Copyright 2008, The Endocrine Society.

2.3.7 5α-DHT increased HERG currents in CHO cells co-expressing HERG K^+ channel and AR45

The data described above indicate that chronic 5α-DHT treatment can increase HERG protein level. Here we employed patch clamp recording to investigate whether androgen treatment also increased HERG K^+ channel function. CHO cells bearing little endogenous potassium currents were chosen for the electrophysiological study. Chronic 5α-DHT treatment increased HERG K^+ current densities (Figure 2-7 A-B), but did not change

current kinetics (insets of Figure 2-7 B). When fitting the activation curves derived from tail current, it was found that chronic androgen treatment did not change voltage dependency of activation ($V_{1/2}$: Con: -20.4±3.4 mV; 5α-DHT: -21.3±2.2 mV, n=8, p>0.05). These data suggest that 5α-DHT increased HERG K^+ current density mainly through the elevated HERG protein levels.

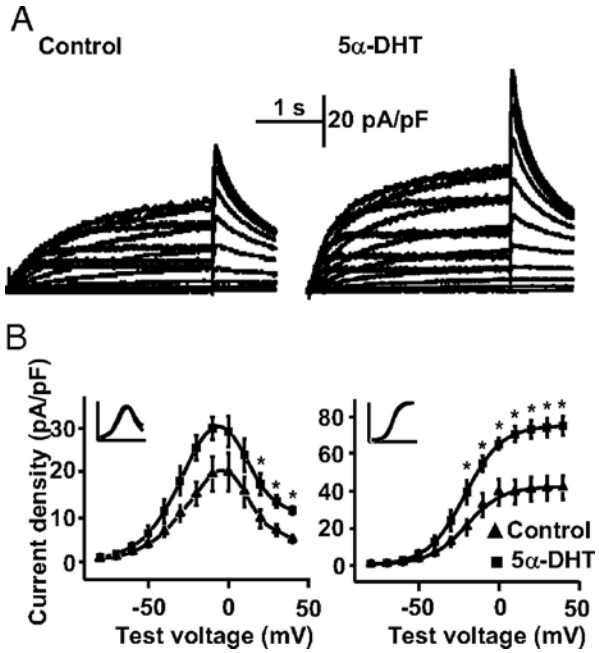

Figure 2-7 Effect of 5α-DHT on HERG K^+ channel activity in CHO cells transfected with HERG K^+ channels and AR45. (A) Representative current tracings showing the effect of 5α-DHT (10 nmol/L, 24 h). Currents were elicited by 4.5 s depolarizing steps to various levels (between 40 and –80mV) followed by repolarizing steps to –70 mV. Scale bars, 20 pA/pF and 1 s. (B). Current-voltage (I-V) relation curve plotted from current measured at the end of the depolarizing test pulses. Inset, data were normalized to unity with and without 5α-DHT. (B) Voltage-dependent activation curves plotted from peak tail currents during a repolarizing step to –70 mV after depolarizing to various voltages. Inset, the same data normalized to the maximum current in the same curve. Square: without 5α-DHT; Triangle: with 5α-DHT treatment. Data are means ± S.E. n = 8 per group. *, $p<0.05$. Copyright 2008, The Endocrine Society.

2.3.8 5α-DHT increased ERG protein level in isolated rabbit cardiac myocytes

To determine whether chronic androgen treatment could also increase native ERG protein level, isolated rabbit cardiac myocytes were cultured in M199 medium in the presence or absence of 5α-DHT for 24 h. It was found that 5α-DHT also significantly increased ERG

protein level (Figure 2-8), which is in agreement with the previous study that in vivo 5α-DHT replacement increased cardiac mycocyte I_{kr} density in orchiectomized male rabbits[116].

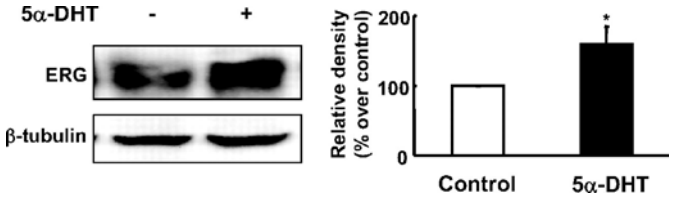

Figure 2-8 Effect of 5α-DHT on ERG protein levels in isolated rabbit cardiac myocytes. Immunoblot analysis shows that treatment with 5α-DHT (10 nM) for 24 h upregulated ERG protein expression in rabbit cardiac myocytes. Data are means ± S.E. n = 4 per group, *, $p<0.05$. Copyright 2008, The Endocrine Society.

2.4 Discussion

AR45 is a naturally occurring splice variant of human androgen receptor [135]. It has a different transcription start point and a novel seven amino-acid long N-terminal extension replaced the region encoded by exon 1 of the AR gene. AR45 shares the same sequence with AR which includes DNA-binding domain, hinge region and ligand-binding domain. The survey on human tissues indicated that AR45 transcript is very high in the heart[135]. In this study, we found that the stimulation of AR45, but not AR, increased HERG protein level. We therefore further examined the underlying mechanisms for the effect of AR45.

The present study demonstrated for the first time that AR45 stimulation increased HERG protein level via a non-genomic effect. We investigated the effect of 5α-DHT on HERG protein level in the presence of cycloheximide, which decreased HERG protein abundance. However, cycloheximide failed to prevent the effect of 5α-DHT. We also noticed that 5α-DHT failed to change the mRNA level of HERG by using RT-PCR analysis. Furthermore, flutamide, a pure antiandrogen which binds to androgen receptor and produces non-genomic effects[141], also increased HERG protein level. Taken together, these data strongly suggest that the augmentation of HERG protein abundance is not via increase of *de novo* protein

synthesis. This is in agreement with the previous finding that AR45 had very weak or no transactivation efficiency[135].

Gong and colleagues demonstrated that HERG proteins, especially the core-glycosylated form of HERG protein, can be degraded in the proteasome[108], while Chapman and colleagues reported that HERG proteins can be degraded in the lysosome[106]. In this study, inhibition of either degradation pathway significantly increased HERG protein levels and attenuated the effect of 5α-DHT. These data indicate that both proteasomal and lysosomal degradation pathways are involved in the stimulatory effect of androgen on HERG abundance. To confirm this finding, we measured HERG protein stability with cycloheximide chase assay and found that 5α-DHT significantly prolonged HERG half-life.

Recent studies demonstrated that ERK1/2 activation regulated protein stability via modulating protein degradation directly or indirectly[147-149]. It was reported that ERK1/2 may phosphorylate MAP Kinase Phosphatase-1 directly and therefore inhibit the degradation of this protein[147]. ERK1/2 can also regulate protein degradation indirectly. For example, activation of ERK1/2 reduced GATA3 degradation by stimulating E3 ligase Mdm2 [149] and promoted p21 protein by enhancing cyclin D1-imposed block[148]. We therefore investigated the involvement of ERK1/2 in the inhibitory effect of androgens on HERG protein degradation. It was found that androgen induced ERK1/2 phosphorylation via AR45 in the same concentration range needed for the effect on HERG protein level. Blockade of ERK1/2 with two specific inhibitors PD98059 and U0126 abolished the augmentation of 5α-DHT on HERG protein level. These data clearly suggest that activation of AR45 in the heart may enhance HERG protein stability via ERK1/2 pathway.

The N-terminal region of AR not only determines the ability of regulating gene transcription but also affects the binding affinity of signaling molecules (i.e. p85α of PI3K) to the receptor[135, 150]. Interestingly, a similar isoform of estrogen receptor α (ERα), ERα46, which lacks AF-1 as AR45, modulates membrane initiated estrogen actions more efficiently than ERα[151], and regulates transcriptional activation less efficiently[152]. These findings and the data from our study suggest that AF-1 in the N-terminal region of sex hormone receptors is critical for the genomic effects of sex hormones. AR and ERα, both of which have AF-1, may contribute more to the genomic effects, whereas AR45 and ERα46 may play a more important role in non-genomic functions within a restricted subset of tissues. The ratios of ERα46/ERα[153-154] and AR45/AR[135] vary in different types of cells and at various developmental stages, which suggests that differential expression of alternatively spliced ER or AR variants may contribute to tissue-specific actions of sex hormones.

Our results also show that long-term 5α-DHT treatment only increased HERG current density but failed to alter HERG current kinetics or voltage-dependence. However, Liu and colleagues found that in vivo 5α-DHT replacement on orchiectomized male rabbits shifted the $V_{1/2}$ of the I_{kr} current to a more negative membrane potential [116]. The discrepancy between our and Liu's data could arise from the effect of 5α-DHT on other isoforms of ERG, β subunits or other impinging signaling pathways not present in our heterologous system. The channel contribution to the cardiac I_{kr} is comprised not only of ERG, but also of ERG1b (N-terminal truncated transcriptional variant of ERG) and β subunits such as MiRP1[51, 80, 155]. To better understand the modulation of androgens on I_{kr}, further studies

are warranted to investigate the effect of androgen on ERG1b and β subunit expression and function.

In this study, we also examined the effect of other sex hormones on HERG protein expression. We found progesterone (30 and 300 nmol/L) and 17-β estradiol (10 and 100 nmol/L) failed to alter HERG protein level. These data suggest that androgens may up-regulate HERG channel protein via stimulation of AR45 specifically.

We also examined the effect of androgens and antiandrogen on HERG protein level. Among them, testosterone is the predominant androgen and can be converted to 5α-DHT and 5β-DHT, and even estrogens. The serum concentration of testosterone is about 10 fold greater than that of 5α-DHT. R1881 is a synthetic androgen with high affinity for the androgen receptor[141]. All of these androgens up-regulated HERG protein abundance in the present study. 5α-DHT was chosen for the rest of the study, because it is a natural, non-aromatisable androgen with high binding affinity and specificity for the androgen receptor. Flutamide has relatively low binding affinity for androgen receptor, and does not possesses any androgenic genomic activity [141, 156]. It has been widely accepted that antiandrogens can antagonize genomic effect of androgens. In the present study, we found that flutamide mimic the effect of 5α-DHT, implying that the effect of androgen on HERG abundance was not a genomic effect. Recent studies showed that similar to androgen, antiandrogen can also activate ERK1/2 [142, 157]. This may explain why flutamide, as an antiandrogen, also upregulated HERG protein expression. The general cardiovascular safety of clinical uses of flutamide may be due, in part, to its lack of inhibition of the non-genomic functions of AR45.

We investigated the molecular mechanism with the heterological expression system and then we also examined the physiological significance in the native tissue. In this study, we found that chronic 5α-DHT treatment significantly increased ERG protein level in rabbit cardiac myocytes, which further confirms the existence of such regulation in cardiac tissue.

In conclusion, our study demonstrates that chronic stimulation of cardiac isoform AR45, but not AR, increased HERG K^+ protein level mainly through stabilizing HERG protein via stimulation of ERK1/2. This finding may provide a molecular mechanism for the gender differences in QTc intervals and drug-induced TdP arrhythmias.

Chapter 3 Progesterone impairs HERG trafficking by disruption of intracellular cholesterol homeostasis

3.1 Introduction

Progesterone (P4) is an important steroid hormone involved in the female menstrual cycle, pregnancy and embryogenesis. Progesterone can enter the cells and activate the progesterone receptor, which in turn modulates the transcription of progesterone response genes. Alternatively, it may stimulate various protein kinases directly or indirectly via plasma membrane receptors. It may activate MAPK to regulate the expression and function of sodium channels [158] and PI3K/Akt pathway to regulate the function of the slow component of the delayed rectifier K^+ current (IKs) and L-type calcium channels [113]. Progesterone may also induce ion channel internalization in oocytes by decreasing the intracellular cAMP level [159]. Moreover, progesterone can also modulate cellular cholesterol homeostasis by affecting cholesterol trafficking, esterification and biosynthesis [160-162].

During the normal menstrual cycle, progesterone levels increase from 0.6-4.5 nmol/L (the preovulatory phase) to 10.5-80 nmol/L (the luteal phase) [163]. It was reported that progesterone at relatively high levels (<100 nmol/L, comparable to that occurring during the luteal phase) had no significant effect on QT interval [164-166]. However, recent studies show that progesterone may shorten the action potential duration or QT interval [167]. This is probably because progesterone at this level (such as during the luteal phase) enhances IKs and inhibits L-type Ca^{2+} currents [113].

If pregnancy occurs, progesterone levels are initially maintained at the luteal level, but may increase to 1 μmol/L at term [168]. Elevated levels of progesterone are important for the implantation of the embryo and the maintenance of a conducive environment for the embryo. At such a high level, the corrected QT (QTc) interval is significantly prolonged [169-170]. This explains why pregnant women are more susceptible to ventricular arrhythmias during

pregnancy, labor and delivery [171-174]. However, it is interesting to note that in the patients with inherited Long QT syndrome (LQTS) the risk for cardiac events is not higher during pregnancy than during other periods in life, whereas the risk markedly increases during the postpartum period [171, 175]. These findings suggest that the mechanisms for pregnancy-induced cardiac events in normal healthy women are different from those in the patients with inherited LQTS.

In the fetus, the progesterone level was reported to be very high (~4.5 μmol/L) in umbilical cord vein during late stages of pregnancy [168]. At this stage, longer QT and QTc intervals were reported in fetal magnetocardiography and non-invasive fetal electrocardiography [176-177]. More importantly, the rate of sudden death is higher in the uterus than at most other times in the human life cycle. Although the major reasons remain unknown, several studies of newborns suggest that QT prolongation and the increased risk of ventricular arrhythmias may account for the significant mortality [178-180]. This may be the same for stillbirth as several case reports indicate that LQTS is one cause for otherwise unexplained fetal demise [181].

The human *ether-a-go-go*-related gene (HERG) K^+ channel, which encodes the α-subunit of the rapid component of the delayed rectifier K^+ current (I_{Kr}), is largely responsible for the repolarization of action potentials in cardiac myocytes. Inherited mutations or drug-induced blockade of the HERG channel prolongs QT intervals and increases the risk of lethal arrhythmia. Trafficking to the plasma membrane is very important for HERG channel and defects in trafficking, caused either by mutation of HERG gene or by drugs, can affect HERG function significantly.

The function of the HERG channel is important for embryo development as impaired HERG channel function not only induces LQTS in embryo, but also affects the structure of the heart [73, 182] and even causes embryonic lethality [182]. However, the effect of progesterone on HERG K^+ channel function remains unclear. Since the progesterone level during late pregnancy is very high, we hypothesized that progesterone may contribute to prolonged QT intervals and the sudden cardiac death in fetus and perhaps in the pregnant women as well. This study was therefore designed to examine the effect of progesterone on protein expression, trafficking and function of HERG K^+ channels in heterologous expression systems and rat neonatal cardiac myocytes.

3.2 Materials and Methods

3.2.1 Materials

PD98059 and LY294002 were from Merck. Simvastatin was from Eurodrug Laboratories (Belgium). All other reagents were from Sigma. Progesterone, cholesterol, and RU486 were dissolved in ethanol as stock solutions. Cycloheximide, PD98059 and LY294002 were dissolved in DMSO as stock solutions. The final ethanol and DMSO concentrations were less than 0.2%. The GFP-Rab9 plasmid was a gift from Dr Suzanne Pfeffer, Stanford University.

3.2.2 Cell culture and transfection

We used HEK293 cells to generate a HERG-HEK293 cell line that stably expresses HERG K^+ channels with a myc tag [183]. HERG-HEK293 cells and CHO cells were cultured in RPMI 1640 (GIBCO, USA) and F-12 (Sigma-Aldrich, USA) cell culture medium respectively, supplemented with 10% fetal bovine serum (FBS, Hyclone) and penicillin-streptomycin (GIBCO, USA) at 37°C in a 5% CO_2 atmosphere. For mechanism studies, inhibitors were added to culture medium 1 hour (unless otherwise stated) before and during progesterone

treatment. For electrophysiological studies, CHO cells were transiently transfected with cDNAs of HERG and GFP by electroporation as described previously [183]. To study the L-type Ca^{2+} current, HEK 293 cells were transiently transfected with human Cav1.2 cardiac splicing form Cav1.2CM (1a-8a-32-33) constructs (1.25 μg), together with β2a (1.25 μg) and α2δ (1.25 μg) using the calcium phosphate transfection method [184]. Lipofectamine 2000 (Invitrogen, USA) was used for the transfection of Myc-Kv1.5, GFP and GFP-Rab9 plasmids.

3.2.3 Isolation of rat neonatal cardiac myocytes

Rat neonatal cardiac myocytes were isolated from 1-2 day Sprague Dawley rats via serial pancreatin (Sigma)/collagenase II (Worthington Biochemical) digestion. Cells were cultured in DMEM medium supplemented with 10% fetal bovine serum, 5% horse serum, and penicillin-streptomycin. To prevent the proliferation of other type of cells, 100 μmol/L BrdU was added in the first 24 hours.

3.2.4 Western blot analysis

Western blot analysis was performed using whole cell lysates as previously described [183]. Anti-myc mouse monoclonal 9E10 and anti-tubulin rabbit monoclonal antibodies were from Sigma. Other antibodies were from Santa Cruz Biotechnology, Inc. All blots illustrated in the figures are representative examples from 4 to 8 independent experiments. ß-Tubulin and HPRT were applied as internal controls to normalize protein loading. The intensity of bands was quantified using LabWorks™ Image Analysis software (UVP).

3.2.5 Confocal microscopy

HERG-HEK293 cells grown on glass coverslips were subject to formaldehyde fixation before indirect immunofluorescent staining. To study the plasma membrane HERG K^+ channel distribution, cells were incubated with anti-Kv11.1 (HERG extracellular epitope,

residues 430-445, between S1-S2 loop) antibody (Alomone Labs) without permeablization, and the primary antibody was detected with Alexa Fluor 488-conjugated anti-rabbit Ig (WGA; Invitrogen). To study the intracellular HERG K^+ channel and ER and Golgi complex localization, cells were permeabilized with Triton X-100 or saponin. Then cells were incubated with anti-c-myc antibody (Sigma) and anti-GM130 (Golgi complex marker) or anti-calnexin antibody (ER marker) (Sigma). Primary antibodies were detected with Alexa Fluor 488-conjugated anti-rabbit Ig and Alexa Fluor 568-conjugated anti-mouse Ig (WGA; Invitrogen). After staining, coverslips were mounted with Prolong Gold antifade reagent (Invitrogen), and confocal images were acquired using Leica TCS SP5 confocal system (Centre for Life Sciences) and Nikon A1R Confocal system (SBIC-Nikon Imaging Centre). Image acquisition and analysis procedures were as previously described [183].

3.2.6 Filipin Staining

HERG-HEK293 cells were fixed with formaldehyde and glycine was used to quench extra formaldehyde. Cells were then stained with filipin (Sigma-Aldrich) and viewed by fluorescence microscopy at magnificent of x20 or x40 times using a UV filter set.

3.2.7 Cellular cholesterol measurement

Amplex®Red Cholesterol Assay kit (Molecular Probes) was used to measure cellular cholesterol level. HERG-HEK293 cells were lysed with reaction buffer containing 0.1 M potassium phosphate, pH7.4, 0.05 M NaCl, 5 mM Cholic acid, 0.1% Triton X-100. Total cellular cholesterol was measured in the presence of cholesterol esterase, which hydrolyzed cholesteryl esters into cholesterol. Free cellular cholesterol was measured in the absence of cholesterol esterase. Bradford assay was used to determine protein concentrations (Bio-Rad).

3.2.8 Patch Clamp recording

Cells grown on cover slips were placed in an acrylic/polystyrene perfusion chamber (Warner Instruments) for electrophysiological measurements. Patch pipettes were pulled and polished to obtain a tip resistance of 2-3 MΩ in the patch clamp solutions. For K^+ channel current recording, the pipette solution consisted of (in mmol/L) KCl 126, $MgSO_4$ 2, $CaCl_2$ 0.5, EGTA 5, Mg-ATP 4, and HEPES 25 (pH7.2, osmolality: 280±10 mOsm), whereas external bath solution consisted of (in mmol/L) NaCl 150, $CaCl_2$ 1.8, KCl 4, $MgCl_2$ 1, glucose 5, and HEPES 10 (pH7.4; osmolality: 320±10 mOsm). For L-type Ca^{2+} current recording, the internal solution (pipette solution) contained (in mM) 138 Cs-$MeSO_3$, 5 CsCl, 0.5 EGTA, 10 HEPES, 1 $MgCl_2$, 2 mg/ml Mg-ATP, pH 7.3 (adjusted with CsOH), whereas the external solution contained (in mM) 10 HEPES, 140 tetraethylammonium methanesulfonate, 5 $BaCl_2$ (pH was adjusted to 7.4 with CsOH and osmolarity to 290-310 with glucose). All experiments were carried out at room temperature (20-22º C). Cells were studied on an inverted microscope (Nikon) equipped with electronic patch-pipette micromanipulators (Burleigh) and epifluorescence optics for GFP transfected cells. The Axopatch-200B patch clamp amplifier and Axopatch 700B amplifier (Axon Instruments) were used for voltage-clamp measurements. The series resistances were about 9 to 10 MΩ. Voltage-clamp protocols were controlled using pClamp9.2 software. To elicit HERG K^+ currents depolarizing voltage pulses were applied to various levels from a holding potential of –70 mV for 4 s followed by stepwise repolarization to –50 mV to measure outward tail currents. Signals were analog-filtered at 2,000 Hz and sampled at 5,000 Hz. Voltage-dependent activation data were fitted to a Boltzmann equation: $I = 1/(1+\exp[(Vh-V)/k])$, where I is the relative tail current amplitude, V is the applied membrane voltage, Vh is the voltage at half-maximal activation, and k is the slope factor. The steady state activation of HERG currents

was studied based on the tail currents. The peak currents were determined by fitting the tail currents with 2-exponential equation and extrapolated back to the moment of repolarization. The values of peak currents were normalized with the maximum current value and fitted with Boltzmann equation to get the steady state activation curve. To study channel deactivation, we activated channels by a voltage pulse to 40 mV, followed by the testing potentials that increased in 10 mV increments from -70 to -40 mV. The deactivation constants (fast and slow time constants) were derived by fitting the currents with a double exponential function.

3.2.9 Statistics

Values presented are as means ± SEM. ANOVA and Tukey's post-hoc tests were employed to assess statistical significance and P-values of < 0.05 were considered to be significant.

3.3 Results

3.3.1 Progesterone impairs the maturation of HERG K^+ channels

The concentration-dependent effect of progesterone on HERG K^+ channel protein expression was first examined. As shown in Figure 3-1 A, there are two forms of the HERG protein in HERG-HEK293 cells. The 135 kD form represents the core-glycosylated protein located mainly in the ER, and the 155 kD form represents the fully-glycosylated protein located mainly in the Golgi complex and plasma membrane. Progesterone at 0.5 μmol/L or higher concentration significantly decreased the amount of the mature (fully glycosylated) form, but had no significant effect on the immature (core-glycosylated) form of the HERG K^+ channel.

In a subsequent time-course study, the significant inhibitory effect on channel maturation was observed after treatment with progesterone for 8 h (Figure 3-1 B). The effect of progesterone was reversible as removal of progesterone for 6 h successfully rescued the

mature form of HERG (Figure 3-1 C). The above data suggest that the effect of progesterone on HERG maturation is both concentration- and time-dependent.

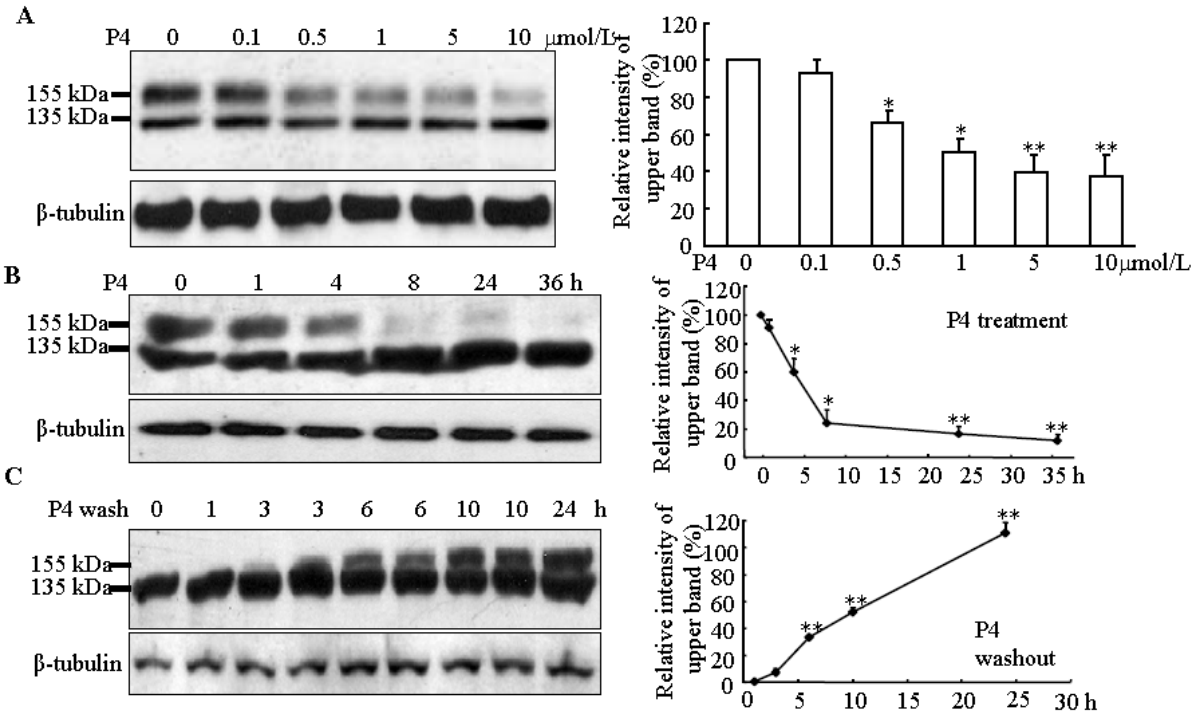

Figure 3-1. Effect of progesterone on HERG protein expression and maturation in HERG-HEK293 cells. (A) Dose-dependent response. Cells were treated with progesterone (0.1-10 μmol/L) for 24 h. n=6. (B) Time-course for the effect of progesterone. HERG-HEK293 cells were incubated with progesterone (5 μmol/L) for different periods. (C) Time-course for the recovery of progesterone effect after washout. n=4. Mean±SEM, * $p<0.05$, ** $p<0.001$ vs P4=0 or Time=0.

3.3.2 Progesterone significantly decreases HERG current density

The effect of progesterone on HERG current was investigated in cell lines stably expressing HERG gene with a whole-cell patch clamp technique. The voltage step protocols are shown in figure 3-2 and described in the figure legend. As shown in Figure 3-2 A-B, treatment with progesterone (0.5-5 μmol/L, 24 h) concentration-dependently decreased current density at the peak of tail current in HERG-HEK293 cells. Acute progesterone treatment (0.05-5 μmol/L) for 15 min failed to affect HERG K^+ current density (data not shown), which is in agreement with the previous study on I_{kr} [113]. These data suggest that the long-term effect of progesterone

was not secondary to its direct interaction with HERG channels. We further confirmed the effect of progesterone on HERG channel current kinetics in CHO cells,

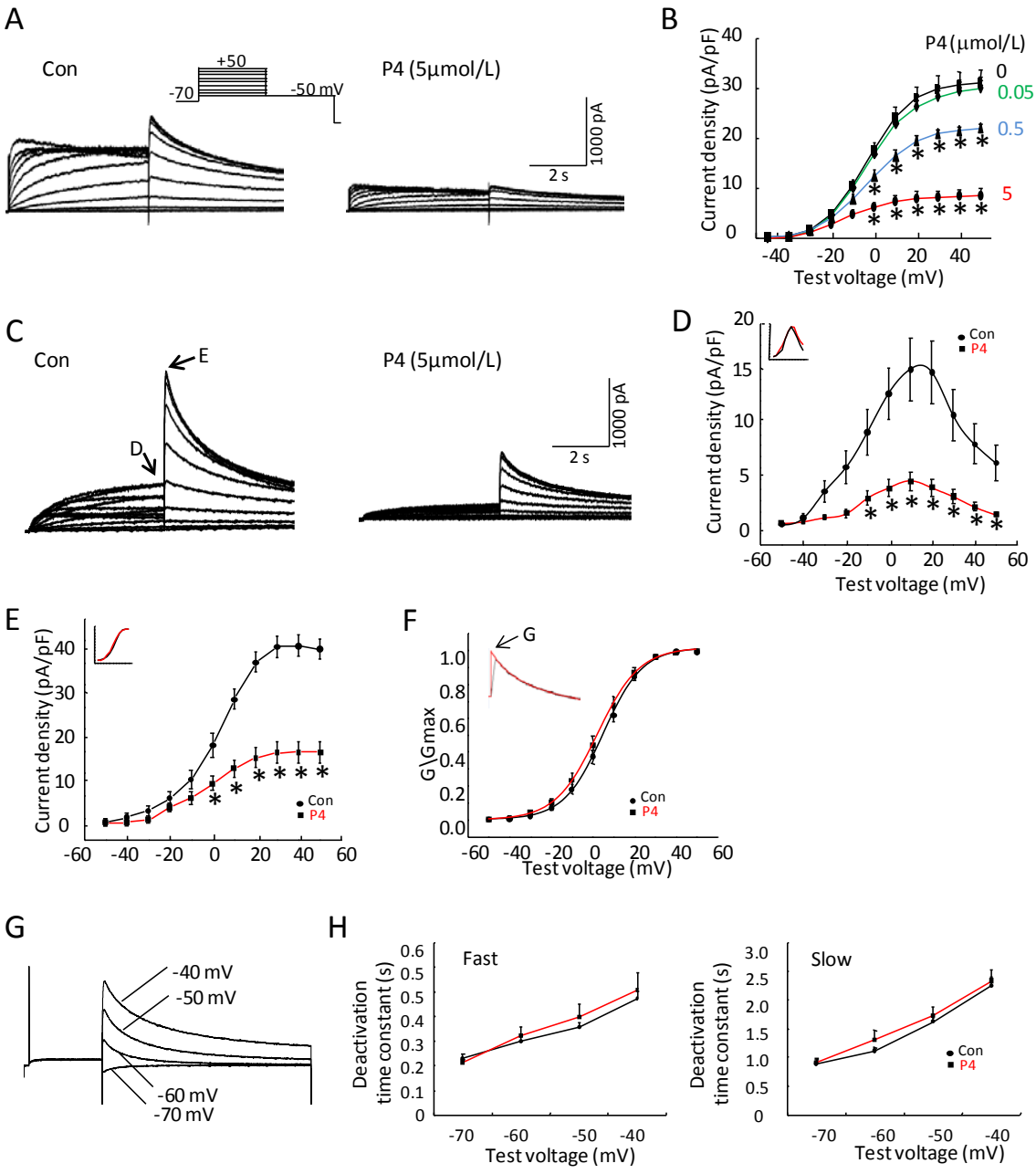

Figure 3-2. Effect of progesterone on HERG K^+ channel function. (A-B): HERG currents measured in HERG-HEK293 cells. (A) Representative recordings showing that the currents were suppressed by progesterone (5 µmol/L, 24 h). (B) Concentration-dependent effect of progesterone (0.05-5 µmol/L, 24 h) on the peak tail currents during a repolarizing step to -70 mV after depolarization to various voltages. (C-H): HERG currents measured in CHO cells. (C) Representative current recordings with or without progesterone treatment. (D) Current-voltage (I-V) relation curve plotted from current measured at the end of depolarizing test pulses. Inset:

Data were normalized to unity with and without progesterone treatment. (E) Voltage-dependent activation curves plotted from the peak tail currents. Inset: Same data normalized to the maximum current within the same curve. (F) Steady state activation curves with or without progesterone treatment. Inset showing measurements of steady-state activation by fitting and extrapolating back to the moment of repolarization (Red line). Currents in (A-F) were elicited by 4 s depolarizing steps to various levels (between -50 and 50 mV) followed by repolarizing steps to -50 mV then to -120 mV. (G-H) Representative recordings and group data showing that treatment with progesterone (5 μmol/L, 24 h) did not affect the deactivation kinetics of HERG channels. Currents were elicited by depolarization of +40 mV followed by repolarization to -70, -60, -50 and -40 mV. Mean±SEM. n=8 cells for each group. * $p<0.05$ vs the corresponding values in the control group (without progesterone treatment).

which express less endogenous potassium current. As shown in Figure 3-2 C-E, progesterone significantly suppressed HERG current density in both current-voltage (I-V) relation curve (Figure 3-2 D) and voltage-dependent activation curve (Figure 3-2 E). When the peak amplitudes of the tail current were normalized, a marginal shift in voltage-dependent activation was observed (Con: 3.8± 0.7 mV; P4: 0.1± 0.8 mV, $p>0.05$, n=8-10, inset of figure 2E). There was no significant change in the slope factors (Con: 10.6± 0.6 mV; P4: 11.5± 0.7 mV). Similar results were observed in the steady state activation curve (Figure 3-2 F). Progesterone treatment had no significant effect on the deactivation of HERG currents (Figure 3-2 G-H). Overall, prolonged progesterone treatment reduced functional HERG K^+ channel density on plasma membrane without affecting HERG K^+ current kinetics.

3.3.3 Progesterone preferentially decreases HERG channel protein in the plasma membrane

Since the fully glycosylated form mainly represents the mature channels expressed in the plasma membrane, we further investigated the effect of progesterone on HERG K^+ channel subcellular compartmentalization by performing immunofluorescence labeling. To detect the surface expression, cells were incubated with anti-Kv11.1 raised against the extracellular loop between S1 and S2 domains. Confocal microscopic examination showed the fluorescent

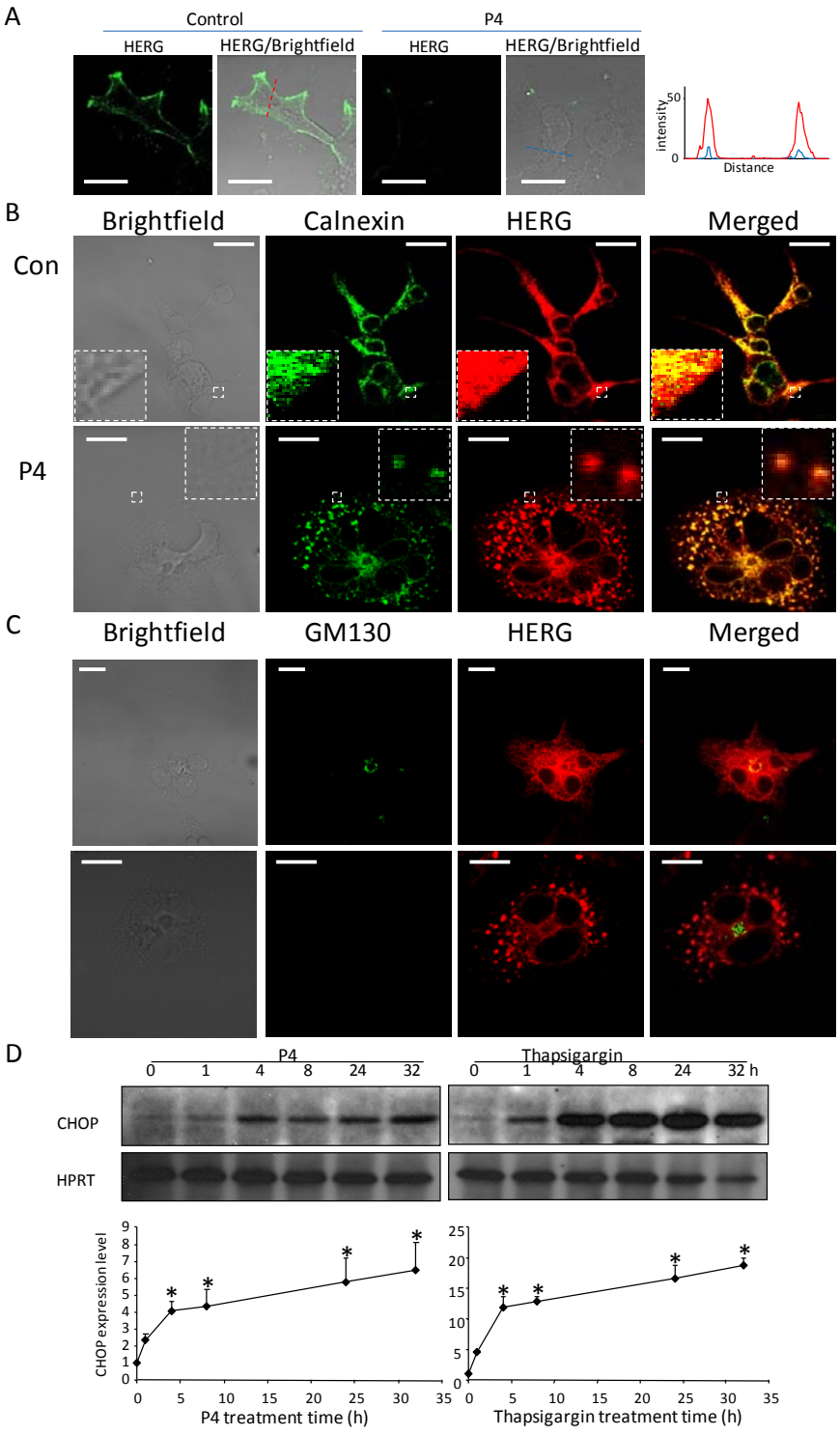

Figure 3-3. Immunofluorescence and Western blotting analysis showing HERG K^+ channel cellular distribution and ER stress caused by progesterone (5 µmol/L, 24h). (A) Plasma membrane HERG K^+ channel proteins determined with an antibody against HERG extracellular domain. Cells were not permeabilized. Right panel shows the fluorescent signal intensities of HERG protein collected from the lines across the cells as indicated in the brightfield pictures. Values were the averages from ten cells in each group. Red: HERG signals in Control;

Blue: HERG signals in progesterone treated cells. (B) Double staining for HERG (red color) with anti-myc antibody and ER marker calnexin (green color). Cells were permeabilized with Triton X-100 before incubation with antibodies. Progesterone treatment caused accumulation of HERG K^+ channel in the dilated ER. Insets, enlarged pictures of the indicated boxes to show the co-localization of HERG and ER. (C) Double staining for HERG (red color) with anti-myc antibody and Golgi complex marker GM130 (green color). All these data are representatives of three to four independent experiments. White scale represents 20 μm length. (D) Both progesterone (5 μmol/L) and thapsigargin (200 nmol/L) increased CHOP expression in a time-dependent manner. Mean±SEM. n=4. * $p<0.05$ vs the corresponding value at time 0.

HERG signals at the cell surface (Figure 3-3 A). The signals were quantified with the software ImageJ as shown in the right panel. It was found that progesterone significantly reduced HERG expression on the plasma membrane (Con: 50.5±7.7 vs P4: 10.4±5.0, $p<0.05$). To further confirm the preferential effect of progesterone, cells were permeabilized and double-stained with anti-myc and anti-calnexin (ER marker) or anti-GM130 (Golgi complex marker) antibodies. Progesterone caused ER dilation (dotted green signal, anti-calnexin), and HERG K^+ channels (red signal) accumulated in the dilated ER (Figure 3-3 B). In contrast, there is no co-localization between the accumulated HERG K^+ channels (red signal) and the Golgi complex (green signal, anti-GM130) (Figure 3-3 C). This result indicates that progesterone treatment may cause ER stress and affect HERG K^+ channel folding and trafficking.

To study progesterone-induced ER stress, we further examined the protein expression of CHOP, a hallmark of ER stress. We found that progesterone increased CHOP protein expression starting from 4 h of treatment in a time-dependent manner (Figure 3-3 D Left panels). This is mimicked by thapsigargin[185], which induces ER stress via blocking ER Ca^{2+}-ATPase and disturbing ER Ca^{2+} homeostasis (Figure 3-3 D right panels). These data clearly suggest that progesterone may induce a HERG trafficking defect via promoting ER stress.

3.3.4 Role of protein kinases in the effect of progesterone on HERG K+ channels

Progesterone regulates the expression and function of sodium and potassium ion channels via activation of protein kinases [113, 158]. The role of protein kinases in the action of progesterone on HERG channels was therefore examined. As shown in Figure 3-4, the effect of progesterone on HERG trafficking was intact in the absence or presence of PD98059 (an ERK1/2-MAPK inhibitor), SB203580 (a p38-MAPK inhibitor), the JNK inhibitor, LY294002 (a PI3K inhibitor) or H-89 (a PKA inhibitor). We previously reported that cAMP may increase both mature and immature forms of HERG protein in HERG-HEK293 cells. We therefore examined whether cAMP can prevent the effect of progesterone. As shown in Figure 3-5, administration of a membrane permeable cAMP analog failed to prevent the progesterone-induced HERG K channel trafficking defect. These data suggest that the effect of progesterone is independent of the activation of these protein kinases.

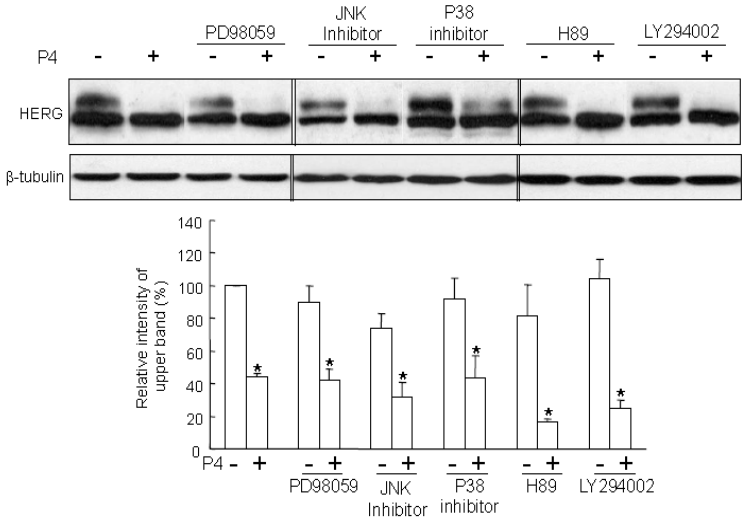

Figure 3-4. Effect of progesterone (5 μmol/L, 24 hours) on HERG channel maturation in the presence or absence of inhibitors of various protein kinases. PD98059 (20 μmol/L, ERK1/2 inhibitor), JNK inhibitor (20 μmol/L), SB203580 (20 μmol/L, p38 inhibitor), H89 (100 nmol/L, PKA inhibitor), and LY294002 (20 μmol/L, AKT/PI3K inhibitor) failed to reverse the effect of progesterone. Mean±SEM. n=5. * $p<0.05$.

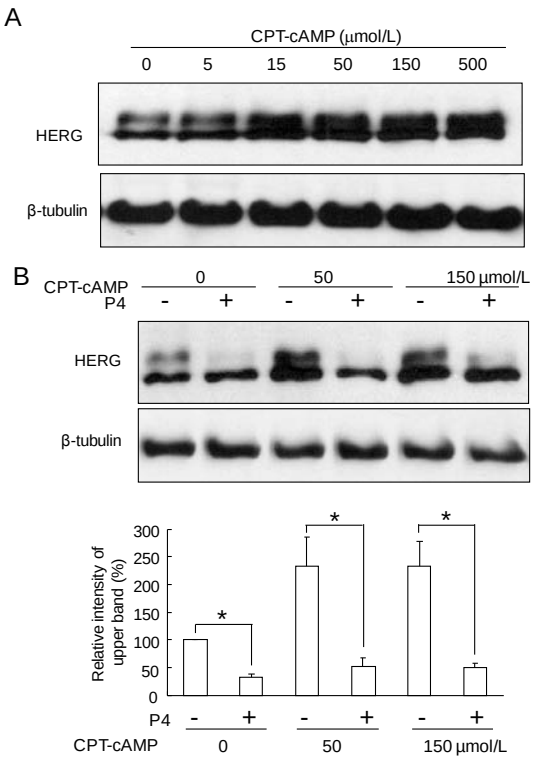

Figure 3-5. Effect of cAMP on HERG channel protein expression in the presence or absence of progesterone. (A) CPT-cAMP (5-500 μmol/L, 24 h) increased HERG protein (both immature and mature) abundance in a dose-dependent manner. Representative Western blots of 3 independent experiments. (B) CPT-cAMP (50 and 150 μmol/L) failed to reverse the effect of progesterone (5μmol/L, 24 hours). Mean±SEM. n=4. * $p<0.05$.

3.3.5 The effect of progesterone is neither progesterone receptor-mediated nor via *de novo* protein synthesis

To examine whether the effect of progesterone is mediated by the nuclear progesterone receptor, RU486, a progesterone receptor antagonist, was used. RU486 decreased the mature form of HERG K^+ channel slightly, but failed to block the inhibitory effect of progesterone on HERG maturation (Figure 3-6 A). These data suggest that the effect of progesterone is not mediated by the nuclear progesterone receptor. This is also supported by our functional data that progesterone decreased HERG K^+ current density in CHO cells (Figure 3-2 C-E), which do not express progesterone receptors [186]. As wide spectrum effects of progesterone are mediated by altering synthesis of proteins, we also examined whether the effect of

progesterone on HERG channels is secondary to *de novo* protein synthesis. Cycloheximide, a protein synthesis inhibitor, was employed. As shown in Figure 3-6 B, cycloheximide significantly decreased the total amount of HERG protein, but failed to prevent/abolish the effect of progesterone on HERG K^+ channel maturation. These results indicate that the effect of progesterone on HERG channel trafficking is neither via stimulation of nuclear progesterone receptor nor via *de novo* protein synthesis.

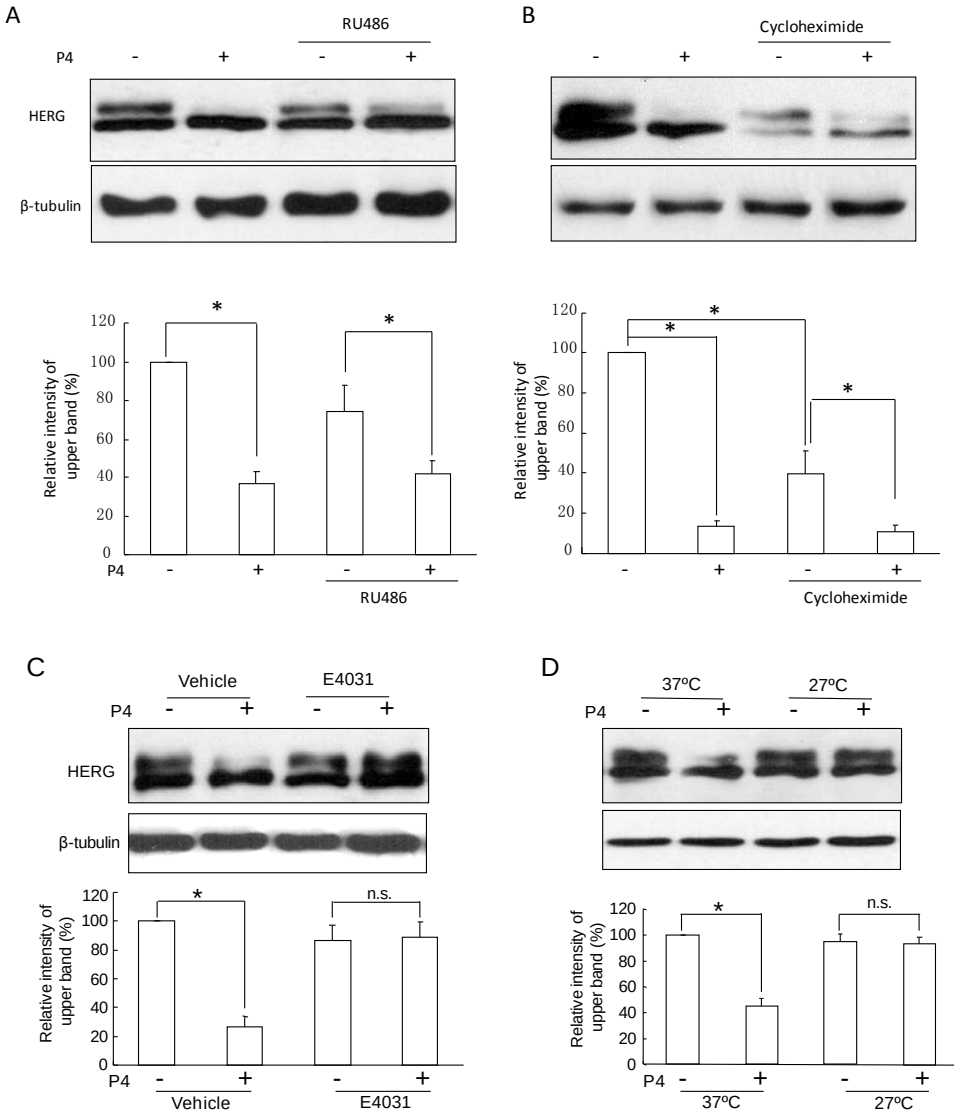

Figure 3-6. Western blotting analysis showing the effect of progesterone (5 μmol/L, 24h) on HERG channel maturation at different situations in HERG-HEK293 cells. (A) The effect of progesterone was receptor

independent. RU486 (10 μmol/L) failed to reverse the effect of progesterone. (B) Effect of progesterone in the presence or absence of cycloheximide (CHX, 40 μmol/L). (C-D) The impaired HERG maturation was rescued by either treatment with 5 μmol/L E-4031 (C) or low temperature (27 ºC) incubation (D). Mean±SEM. n=4-5. * $p<0.05$, n.s.: not significant.

3.3.6 The effect of progesterone is reversed by a HERG channel blocker and low culture temperature

Both E-4031, a HERG channel blocker, and low temperature (27 ^{o}C) improve the proper folding of HERG channel in ER [90]. As shown in Figure 3-6 C-D, both maneuvers rescued the HERG channel trafficking-defect caused by progesterone. These data imply that progesterone may affect HERG channel folding in ER and/or block its trafficking to Golgi complex.

3.3.7 Effect of progesterone on cholesterol level and distribution

Protein folding/trafficking can be directly or indirectly affected by cholesterol [187-190]. We first examined the role of cholesterol in the regulatory effect of progesterone on HERG K^+ channel maturation. The free cholesterol in the cells was stained with filipin. As shown in Fig 3-7 A, progesterone caused free cholesterol accumulation in the cytosol in a concentration-dependent manner (1-5 μM). 2-hydroxypropyl-β-cyclodextrin (HPCD), a sterol-binding agent, redistributed cholesterol in the compartments of cells (Figure 3-7 B), which is consistent with previous reports [191-192].

The effect of progesterone on cholesterol content was also examined. As shown in Figure 3-7 C, progesterone had no significant effect, whereas simvastatin (10 μM, an inhibitor of *de novo* cholesterol synthesis) significantly decreased free and total cholesterol levels in HERG-HEK293 cells. These data suggest that progesterone can only affect the distribution of cholesterol, but had no effect on the amount of total- and free- forms of cholesterol.

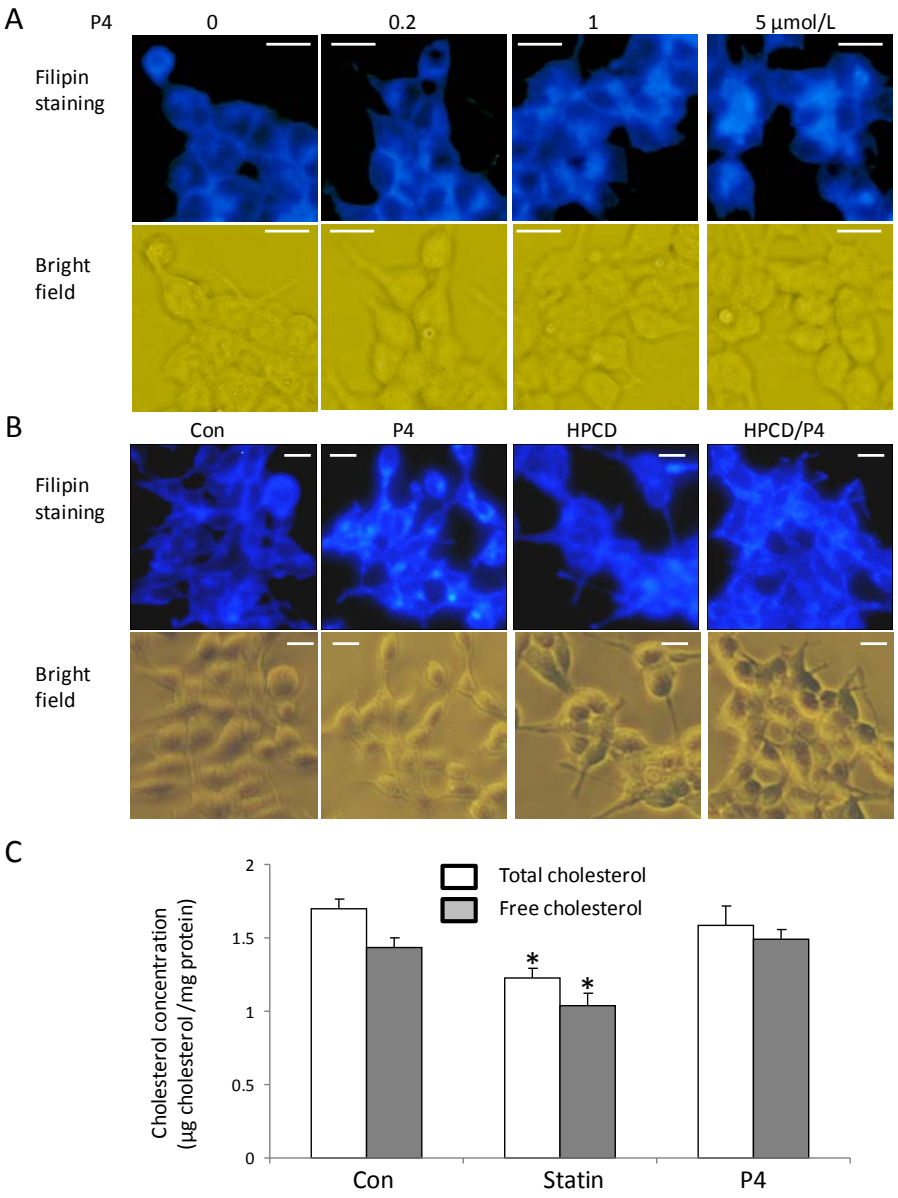

Figure 3-7. Effect of progesterone on the distribution and level of cholesterol in HEK293 cells. (A-B) Filipin staining of intracellular free cholesterol. (A) Progesterone induced accumulation of cholesterol in a dose-dependent manner. (B) Sterol binding agent 2-hydroxypropyl-beta-cyclodextrin (HPCD, 10 mg/ml) abolished progesterone (5 µmol/L, 24 hours)-induced cholesterol accumulation. Photomicrographs were taken on an inverted microscope. This result represents three independent experiments. White scale bar represents 15 µm. (C) Cellular cholesterol levels were measured with Amplex®Red Cholesterol Assay. Cells were treated with simvastatin (10 µmol/L) or progesterone (5 µmol/L) for 24 hours. Mean±SEM. n=6. * $p<0.05$ vs the corresponding value in the control group.

3.3.8 Progesterone blocks HERG channel trafficking via disturbing intracellular cholesterol homeostasis

To investigate whether progesterone-induced accumulation of cholesterol is involved in the HERG trafficking defect, HER channel protein levels were determined in WT and mutant channels with and without HPCD treatment. Western blotting data showed that HPCD reversed the effect of progesterone on HERG channel maturation, but had no effect on that of V630A, a HERG trafficking mutant (Figure 3-8 A). These results suggest that intracellular cholesterol is involved in the mediation of WT HERG channel trafficking defect by progesterone but not the mutant HERG channel.

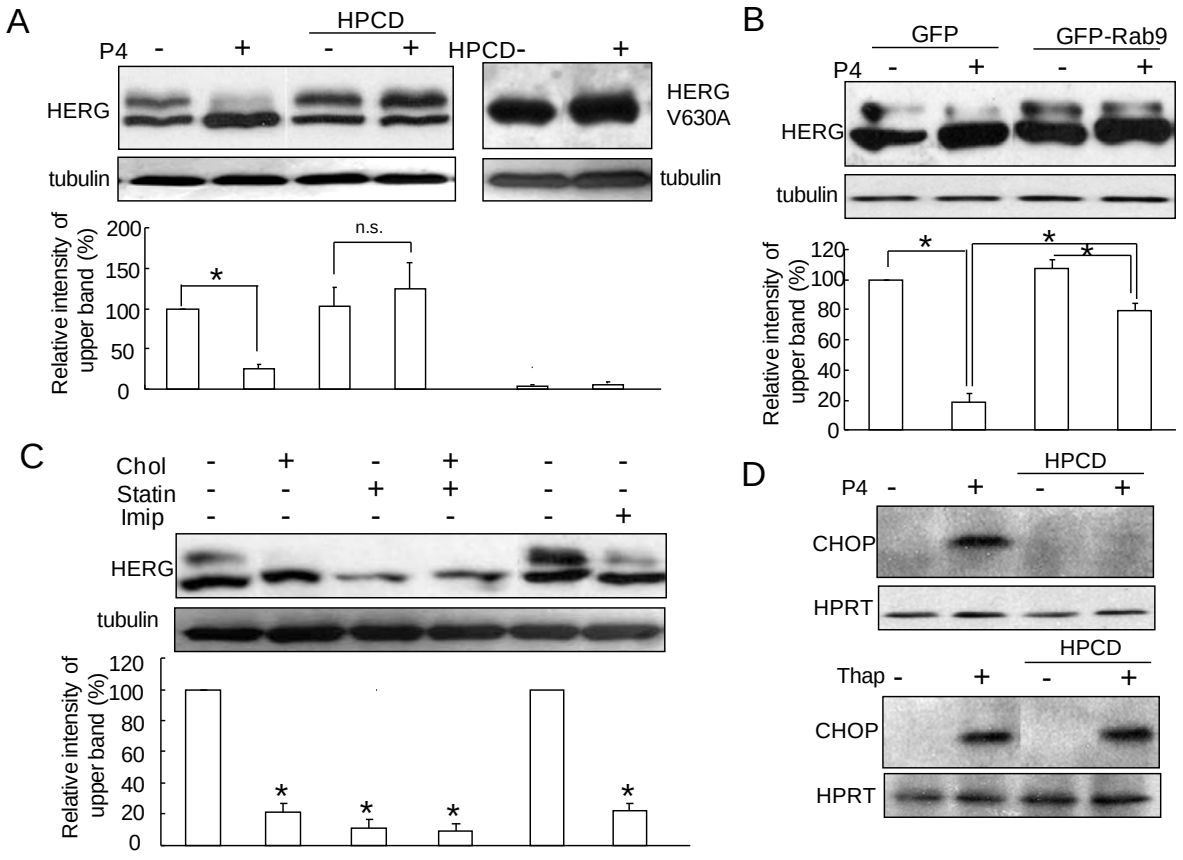

Figure 3-8. Progesterone impaired HERG maturation via disrupting intracellular cholesterol homeostasis. (A) HPCD (10 mg/ml) rescued the HERG trafficking defect caused by progesterone (5 μmol/L, 24 hours), but not that caused by the V630A HERG mutant. n=4. (B) Over-expression of Rab9 partially rescued the progesterone(5 μmol/L, 24 hours)-induced HERG trafficking defect. n=6. (C) Effect of simvastatin (statin, 10

μmol/L), cholesterol (Chol, 20 μg/ml) or imipramine (Imip, 30 μmol/L) on HERG maturation. n=5. (D) HPCD (10 mg/ml) abolished the upregulated CHOP expression induced by progesterone (5 μmol/L), but not by thapsigargin (Thap, 200 nmol/L). The data represent three independent experiments. Mean±SEM. * $p<0.05$. n.s.: not significant.

Rab9 GTPase is important for cholesterol trafficking and overexpression of Rab9 prevents cholesterol accumulation [193]. We found in the present study that over-expression of Rab9, which itself had no effect on HERG trafficking, but partially reversed the HERG trafficking defect caused by progesterone (Figure 3-8 B). These data confirm that cholesterol accumulation is responsible for the progesterone-induced HERG trafficking defect.

To confirm these findings, we decreased the cholesterol level with simvastatin, accumulated free cholesterol in the late endosome/lysosome with imipramine, or delivered a large amount of exogenous cholesterol into the cells. We found that all these treatments mimicked the effect of progesterone. In addition, simvastatin failed to reverse the effect of cholesterol (Figure 3-8 C). These data suggest that not only the cholesterol level, but also the distribution of free cholesterol, is important for HERG maturation.

We further tested the involvement of cholesterol in progesterone-induced ER stress. As shown in Figure 3-8 D, HPCD reversed progesterone-induced up-regulation of CHOP expression, but failed to affect thapsigargin-induced CHOP expression. These data further indicate that progesterone may impair HERG maturation by disturbing intracellular cholesterol homeostasis and the subsequent ER stress.

3.3.9 The specificity of progesterone on HERG K^+ channel trafficking

In this series of experiments, we first studied whether progesterone has a similar effect on L-type $Ca_V1.2$ calcium channels. The human cardiac $Ca_V1.2CM$[184], and its accessory subunits, β_{2a} and $\alpha_2\delta$, were transfected into HEK 293 cells. Two protocols, IV protocol (Figure 3-9 A) and tail protocol (Figure 3-7B), were employed to record I_{Ba} carried by Ba^{2+} through the

$Ca_V1.2$CM channels. As shown in the right panels of Figure 3-9 A-B, the current densities recorded with the two protocols were not affected by progesterone (5 μM, 24 h). The effect of progesterone on endogenous voltage-gated K^+ current (I_{Kv}) was also examined. As shown

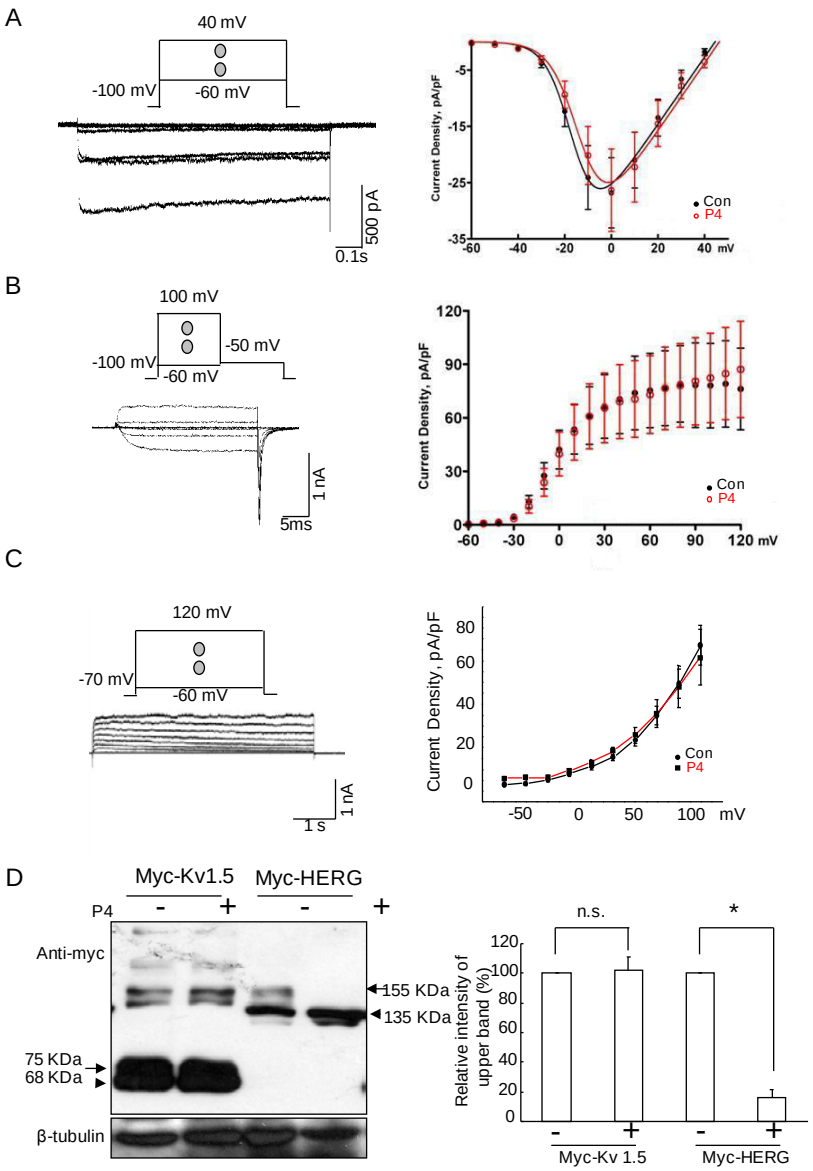

Figure 3-9. Effects of progesterone (5 μmol/L, 24 h) on currents of Voltage-gated L-type Ca^{2+} channels, endogenous K^+ channels and trafficking of Kv1.5 channels. (A-B) Effect of progesterone on L-type Ca^{2+} current density. (A) Current-voltage (I-V) relation curve plotted from peak currents at different voltages. Cells were activated from the holding potential (-100 mV) via a series of depolarizing pulses (-60 to 40 mV) for 900 ms. n=9-12. (B) I-V curve plotted from the peak tail currents. Cells were activated via a series of depolarizing pulses (-60 to 100 mV) for 20 ms, and the tail currents were recorded at the voltage of -50 mV. n=7-12. (C) Effect of progesterone on endogenous voltage-gated K^+ currents of HEK293 cells. I-V relation curve plotted

from the currents measured at the end of depolarizing test pulses. Cells were activated from the holding potential (-70 mV) via a series of depolarizing pulses (-60 to 120 mV) for 5 s. n=9. (D) Effect of progesterone on the trafficking of Kv1.5 and HERG. Both myc-Kv1.5 and myc-HERG were transiently transfected into HEK293 cells. Cells were treated with progesterone or vehicle for 24 h. Anti-myc antibody was used to detect both myc-Kv1.5 and myc-HERG. For these two potassium channels, the upper bands (arrows) indicate the mature or fully-glycosylated form, and the lower bands (arrowheads) indicate the immature or core-glycosylated form. Mean±SEM. n=5. * $p<0.05$.

in Figure 3-9 C, progesterone had no significant effect on current density of endogenous I_{Kv}. Kv1.5 channel undergoes similar glycosylation and trafficking to HERG. The fully-glycosylated form (75 kDa) mainly represents the plasma membrane channel, whereas the core-glycosylated form (68KDa) mainly represents the immature form in ER [194]. As shown in Figure 3-9 D, treatment with progesterone (5 μM) for 24 h only impaired the trafficking of HERG, but had no effect on that of Kv1.5 K^+ channels. Taken together, these results demonstrate that progesterone specifically induces the trafficking defect of HERG K^+ channel, but not $Ca_V1.2$ channels and Kv1.5 K^+ channels.

3.3.10 Progesterone impaired the maturation of ERG K+ channels in rat neonatal cardiac myocytes

To test whether progesterone affects ERG K^+ channels in the native cells, rat neonatal cardiac myocytes were isolated and cultured with progesterone. As shown in Figure 3-10, there are two forms of ERG protein (160 KDa and 120 KDa), which are consistent with the mature and immature forms of rat ERG1a as previously reported [155]. We found that treatment with progesterone (5 μM) for 24 h significantly decreased the mature form of the ERG K^+ channel, suggesting that progesterone may also impair the maturation of ERG channels in the native cardiac tissue.

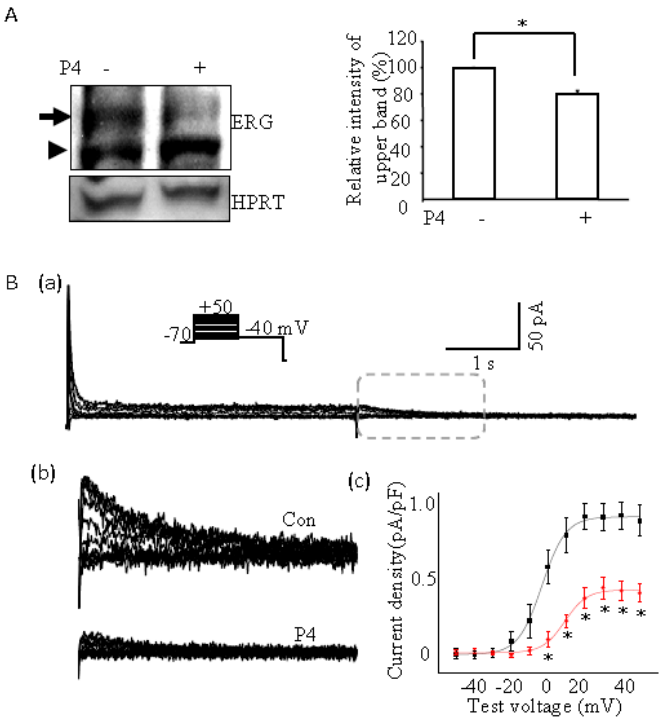

Figure 3-10. Effect of progesterone on ERG protein expression (A) and currents (B) in rat neonatal cardiac myocytes. One day after isolation, rat neonatal cardiac myocytes were treated with progesterone (5 μmol/L, 24 h). (A) Western blots showing protein expression. The upper band (arrow pointed, around 160 kD) is the mature form of the ERG channel, whereas the lower band (arrowhead, around 130 kDa) is the immature form. Mean±SEM. n=8. * $p<0.05$. (B) ERG/IKr recording. (a) Representative current tracings recorded from rat neonatal cardiac myocyte. Inserts show the recording protocol and current and time scales. (b) Enlarged tracings of the indicated box in (a) to show that rat ERG currents were suppressed by progesterone (5 μmol/L, 24 h); (c) Voltage-dependent activation curves plotted from peak tail currents during a repolarizing step to -40 mV after depolarizing to various voltages. ■,control, n=26; ●, progesterone treatment, n=17. Mean±SEM. * $p<0.05$.

3.4 Discussion

HERG trafficking defects are one of the main causes of long QT syndrome (LQTS). Trafficking can be impaired by mutations of the channel [27] or induced by drugs such as probucol, cardiac glycosides, fluoxetine, norfluoxetine, pentamidine, arsenic trioxide and celastrol [96, 194]. We report here that a HERG trafficking defect can also be induced by excessive progesterone hormone. The impaired HERG trafficking may, in turn, induce imbalance of heart electrical stability and therefore development of ventricular arrhythmias. This may suggest a mechanism to explain why the corrected QT intervals are longer in pregnancy and why women at late pregnancy are more susceptible to ventricular arrhythmias.

Since progesterone may not be able to further impair the defective trafficking of the mutated HERG channels in the inherited LQTS patients, our findings may also explain why the incidence of arrhythmias is not higher in pregnancy than in other periods in the life of these patients [165, 175].

During the late phase of pregnancy, the progesterone level in fetal circulation (~4.5 μmol/L) is much higher than that in maternal blood (1 μmol/L). This may imply that progesterone has greater impact on the fetal heart and could explain the higher rate of sudden death in the uterus than at most other times in the human life cycle.

Apart from the Western blots and patch clamp data, the impaired HERG protein trafficking by progesterone was further confirmed with confocal microscopy. Progesterone preferentially decreased membrane protein expression. More importantly, progesterone treatment induced ER stress and dilation. Immature HERG protein may be stuck in the dilated ER. This may prevent the maturation and trafficking of HERG into Golgi and plasma membrane.

To investigate the mechanism, we first examined whether the effect of progesterone is mediated by nuclear progesterone receptors. We found that RU486, a progesterone receptor blocker, failed to reverse the effect of progesterone on HERG K^+ channel trafficking. In addition, the inhibitory effect of progesterone on HERG currents in CHO cells, a cell line without expression of progesterone receptor [186], further supports the hypothesis that the effect of progesterone is nuclear receptor-independent. In addition, progesterone did not decrease the total amount of HERG protein. Cycloheximide, a protein synthesis inhibitor, decreased total protein expression but failed to block the progesterone effect. These results indicate that

the effect of progesterone is not via altering protein synthesis. The role of protein kinases was also investigated and we found that the effect of progesterone is not secondary to activation of MAPK, PI3K/Akt, cAMP or PKA.

Cholesterol homeostasis is very important for protein folding and trafficking. Cholesterol may affect membrane protein folding either directly or indirectly. For example, nicotinic acetycholine receptor (nAChR) contains internal binding sites for cholesterol and cholesterol binding stabilizes nAChR protein structure. Overloading of free cholesterol may also directly cause ER stress and ER dilation [195] and therefore impairs protein folding. We therefore examined whether the progesterone-induced HERG trafficking defect involves cholesterol. We found in the present study that progesterone induced intracellular free cholesterol accumulation, which further induces ER stress and ER dilation. Since progesterone can insert into lipid bilayers and perturb membrane function and lipid mobility [196], direct interaction between progesterone and lipids may be responsible for the action of progesterone on intracellular cholesterol homeostasis. HPCD is a sterol-binding agent, which redistributes cholesterol in the cellular compartments. HPCD also can disrupt membrane lipid rafts and affect functions of membrane receptors, ion channels, transporters, protein kinases located/related with lipid rafts. We found that HPCD abolished the effect of progesterone on cholesterol homeostasis, ER stress and HERG trafficking. Rab9 is important for cholesterol trafficking. Overexpression of Rab9 prevents cholesterol accumulation. We found in this study that Rab9 also reversed HERG trafficking defects. These data confirm that the progesterone-induced HERG trafficking defect is secondary to the accumulation of cholesterol. In addition, disrupting intracellular cholesterol homeostasis with simvastatin, imipramine or exogenous delivery of cholesterol mimicked the effect of progesterone on

HERG K^+ channel trafficking. Thus, impaired cholesterol processing (either high levels, low levels or disrupted distribution) may contribute to HERG K^+ channel defects caused by progesterone.

The mechanisms underlying cholesterol-induced ER stress are still not clear. It has been reported that free cholesterol accumulation in the ER membrane activates the unfolded protein response (UPR) [197]. The UPR may induce ER-phagy, which selectively sequesters and tightly packs ER membranes into autophagosomes [198]. In ER, cholesterol is also an important factor for ER-Golgi membrane transport. Sterol depletion in the ER inhibits the ER-to-Golgi transport of secretory cargo membrane protein ts-O45-G, vesicular stomatitis virus glycoprotein and scavenger receptor A [187-188]. In addition, inhibition of the early stage of *de novo* cholesterol synthesis can also affect isoprenoid intermediates, which are very important for the membrane anchoring and activation of small G proteins such as Ras, Rho and Rab. The dysfunction of these G proteins can affect protein trafficking [189-190]. More studies are warranted to examine how exactly cholesterol regulates ER function and ER stress.

In summary, progesterone may disturb intracellular cholesterol homeostasis and block HERG channel trafficking, which may prolong the QT intervals of both mother and fetus and increase the risk of developing ventricular arrhythmias. Our study may reveal a new mechanism for pregnancy-associated LQTS and provide new approaches to prevent ventricular arrhythmias and sudden death.

Chapter4 GENERAL DISCUSSION AND CONCLUSION

4.1 General discussion

Female gender is a risk factor for drug-induced cardiac arrhythmia[110], with an approximately 2:1 preponderance of women to men developing *torsade de pointes* (TdP) arrhythmias in response to similar concentrations of a wide variety of drugs [120]. The international LQTS registry reported that females had high risk of encountering first cardiac event between age 15 and 40 compared with the males at the same age[199].

Several lines of evidence indicate that androgen plays important roles in this gender difference in arrhythmia. Clinical studies indicate that the gender difference in QTc intervals can only be observed after puberty[122]. Adult men have high androgen level (testosterone: 16.8±6.1 nmol/L; 5α-DHT: 1.5±0.8 nmol/L)[119], shorter QTc intervals[122, 200] and lower incidence of cardiac event[200]. In contrast, adult women have lower androgen level (testosterone: 0.6-2.5 nmol/L; 5α-DHT: ~0.02 nmol/L)[118], longer QTc intervals[122] and high frequent occurrence of self-terminating TdP[199]. In addition, cardiac repolarization periods in castrated men and normal women are longer than those in normal men and women with virilization who have higher androgen concentration[124]. Moreover, testosterone replacement therapy restores male ECG patterns of repolarization in the castrated men[124]. This is also supported by animal experiments which show that androgen replacement treatment also restores the prolonged QT intervals in orchiectomized male rabbits [116]. All these experiments suggest that androgen, but not estrogen, is responsible for the different QT intervals and incidence sof cardiac events between men and women.

Among cardiac ion currents, HERG K^+ channel current/I_{kr} is critic for the repolarization of cardiac myocytes. Malfunction of HERG K^+ channel is one of main causes for LQTS. Several findings indicate that the HERG K+ channel current/I_{kr} can be the target of androgen and this modulation may be a potential underlying mechanism for the gender difference in arrhythmia. Rabbit is a good animal model for studying the gender difference in LQTS, as this difference can be observed in rabbit as human beings. In rabbit, it was found that I_{kr} density is high in male cardiac myocytes than in female[117]. Androgen treatment increases I_{kr} density with no changes on HERG mRNA level[116]. However, the molecular mechanism of this modulation is unclear yet.

One of the aims of this study is therefore to investigate the underlying mechanism of androgen modulation on HERG K^+ channel/ I_{kr}. The unexpected result is that N-terminal truncated variant androgen receptor (AR45) but not the full length androgen receptor (AR) is involved in the androgen induced HERG channel protein augmentation. In the heart, the expression level of AR45 is much higher than AR. AR45 has very weak or no transactivation efficiency compared with AR, partially because AR45 lacks activation function-1 domain. In this study, we found that the stimulation of AR45 didn't affect the transcription level of HERG channel, which is consistent with previous study that androgen treatment increased I_{kr} without changes on HERG mRNA level. My further studies indicate that the stimulation of AR45 can stabilize HERG protein. This is a new mechanism for the modulation of HERG channel protein. Taken together, the stabilization of HERG channel proteins can be modulated by HERG gene mutation[82, 107], HERG protein glycosylation[91], ceramide[106], extracellular potassium ion[28, 107, 109] and androgens (this work).

My study also shows that estrogen and progesterone (for non-pregnancy state) do not change HERG channel protein level. For the acute effect, sex hormones (androgen, estrogen and progesterone) have weak or no effect on HERG channel function [112-113, 201]. Taken together, androgen but not estrogen and progesterone, stabilizes HERG channel protein via stimulation of AR45, which may contribute to the gender difference in LQTS.

Other cardiac ion currents may also be modulated by sex hormones and the modulations may affect cardiac repolarization. Furukawa and his colleagues reported that acute treatment with sex hormones potentiated I_{ks} and inhibited $I_{Ca,L}$ [112-113, 201]. The mechanistic studies show that acute treatment with all these three sex hormones can potentiate I_{ks} via different mechanisms. While androgen or progesterone can potentiate I_{ks} and attenuate $I_{Ca,L}$ via AKT/NOS signaling pathway[112-113]. Estrogen potentiated I_{ks} via estrogen receptor[201]. However, as all these sex hormones have similar effect on I_{ks} and $I_{Ca,L}$, this modulation may not contribute to gender difference in LQTS.

Compared with androgen and estrogen whose levels are relatively low (nmol/L) at physiological situation, progesterone can reach very high concentration (μmol/L) in pregnancy and plays some unique roles in human life. Progesterone level may increase from 0.6-4.5 nmol/L during the preovulatory phase to 10.5-80 nmol/L during the luteal phase[163], which is important for embryo implantation. During the pregnancy, progesterone level increases steadily and may reach 1 μmol/L in mother's blood and 5 μmol/L in embryo before the delivery[168]. This high concentration of progesterone is important for maintaining conducive environment for embryo.

However, my study indicates that the very high level of progesterone may be harmful for the embryo as it blocked HERG channel trafficking. HERG channel not only plays important roles in adult but also in the embryo. Malfunctions of HERG channel in the embryo not only induce fetal LQTS, but also induce hypoxia in the embryo, which in turn affect the normal development[182]. It was found that super high progesterone level in embryo is related with high chance of emergency cesarean section[168]. The authors suggested that the hypoxia environment for the embryo may stimulate progesterone secretion. My study indicates that the increased progesterone may disrupt HERG K^+ channel trafficking and affect heart function, which may worsen the hypoxia situation.

In the present study, the data from western blot, immunostaining and patch clamp recording suggest that chronic progesterone treatment inhibited HERG K^+ channel trafficking. The defect of HERG K^+ channel trafficking can be rescued by lower temperature incubation or specific HERG K^+ channel blocker. These data suggested that progesterone may impair HERG K^+ channel folding as lower temperature can prolong the folding process and facilitate HERG K^+ channel proper folding and channel blocker can bind the pore region of the channel and stabilize channel conformation. The confocal images show that progesterone treatment blocked HERG K^+ channel trafficking and accumulated HERG K^+ channels in the dilated ER. As there were changes on the ER morphology, we tested whether progesterone treatment induced ER stress with a hall marker CHOP. Progesterone increased CHOP expression in a time-dependent manner which indicates progesterone treatment can induce ER stress.

As a steroid hormone, progesterone has a variety of targets in the cells. The classical one is the nuclear progesterone receptors, which can trigger a set of gene transcription up on the

progesterone treatment. However, the data from progesterone receptor negative cells and progesterone receptor inhibitor suggested that progesterone impaired HERG K^+ channel trafficking without the involvement of nuclear progesterone receptor. Progesterone may activate numerous protein kinases and impair HERG K^+ channel trafficking. In this study, we employed pharmacological tools to examine the involvement of protein kinases, and found all of the protein kinase inhibitors used here failed to block progesterone induced HERG K^+ channel trafficking.

Progesterone can also impair intracellular cholesterol homeostasis by affecting cholesterol trafficking, esterification and biosynthesis[160-162]. In this study, I found that progesterone changed intracellular cholesterol distribution in a dose-dependent manner. Sterol binding reagent HPCD redistributed intracellular cholesterol, attenuated progesterone induced ER stress and HERG K^+ channel trafficking defect. Intracellular cholesterol trafficking can be facilitated by small GTPase as Rab9[193]. In this study, transfection with Rab9 attenuated the effect of progesterone on HERG K^+ channel trafficking. In addition, the defect of HERG K^+ channel trafficking was mimicked by impairing intracellular cholesterol homeostasis as decreasing the cholesterol level with simvastatin, accumulating free cholesterol in the late endosome/lysosome with imipramine, or delivering a large amount of exogenous cholesterol into the cells. Taken together, these data indicate that the progesterone impaired intracellular cholesterol homeostasis, induced ER stress and defected HERG K^+ channel trafficking.

4.2 Conclusion and Perspectives

This thesis examined how sex hormones regulate HERG K^+ channels with a combination of pharmacological manipulations, confocal microscopy, molecular biology and electrical physiology. The data presented in this thesis demonstrate for the first time that (i) the

stimulation of AR45 by androgen can stabilize HERG K^+ channel protein via ERK1/2 signaling pathway; (ii) progesterone impairs HERG K^+ channel protein trafficking via disturbing intracellular cholesterol homeostasis; (iii) chronic estrogen treatment have no effect on HERG K^+ channel protein level. This study not only provides a molecular mechanism for the sex differences in QT intervals and drug-induced arrhythmias but also explains the longer QT interval during late pregnancy and the high risk of arrhythmias development in the fetus

In general, the significant contribution of this thesis may include that:

- prolonged androgen treatment stabilizes HERG channel protein. These findings may provide an important mechanism for gender difference of Long QT Syndrome.
- cardiac isoform AR45, but not full length AR, is responsible for the effect of androgen on HERG channel proteins. These data indicate that different androgen receptor isoforms may play different physiological roles.
- prolonged progesterone treatment blocks HERG channel protein trafficking. These findings may explain why QT interval is prolonged and why the incidence of cardiac events is higher during the late pregnancy.
- disruption of intracellular cholesterol may block HERG channel protein trafficking. This may open up a new area for unveiling cholesterol related arrhythmia.

Although the full length of HERG protein is the dormant and functional subunit for I_{kr}, it will be interesting to study the effect of sex hormones on two splice variants of HERG protein, N-terminal splice variant (HERG1b)[155, 202] and C-terminal splice variant ($HERG_{USO}$)[203], which were discovered recently. Both HERG1b and $HERG_{USO}$ can be detected in the heart and can modulate HERG current kinetics when co-expressed in heterological expression system[204 203].

In addition, due to the time limitation, only the ERG protein expression was examine in the native cardiac myocytes. Further studies on ERG current and other cardiac ion currents in the native cardiac myocytes are needed for better understanding the modulation of sex hormones on heart function.

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